THE UNIVERSITY OF MANITOBA

STUDIES OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN A HIGHLY HYDROXYUREA RESISTANT MOUSE CELL LINE AND INHIBITION OF RIBONUCLEOTIDE REDUCTASE BY GOSSYPOL

By

© Arthur K.M. Chan

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements for the Degree

Master of Science

Department of Biochemistry

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ABSTRACT

Ribonucleotide reductase catalyzes the reaction in which ribonucleotides are reduced to deoxyribonucleotides, the precursors of DNA synthesis. The mammalian enzyme is highly regulated, and consists of two non-identical subunits often called M1 and M2. The antitumor agent hydroxyurea is a specific inhibitor of ribonucleotide reductase, and was used in a step-wise drug selection procedure, to isolate a mouse L cell line capable of proliferating in the presence of 5 mM hydroxyurea, and was about 30-fold more drug resistant than wild type cells in colony-forming ability experiments. The hydroxyurea resistance characteristics of this cell line, called LHF, was stable for at least 40 passages in the absence of the selective agent. LHF cells contained elevated ribonucleotide reductase activity with an apparent wild type sensitivity to hydroxyurea and dATP, a negative allosteric effector of enzyme activity. Furthermore, ribonucleotide reductase enzyme from both wild type and LHF cells showed similar Km vales for the substrates CDP and ADP. The V_{max} values for the LHF cells were 5 and 3 fold higher than the wild type cells for the substrates CDP and ADP respectively. Interestingly, the increase in enzyme activity in LHF cells was dependent upon whether the cells were previously cultured in the presence of hydroxyurea, and varied between about 5-fold when cells were grown in the absence of drug for two weeks to about 20-fold when grown in the presence of 5 mM hydroxyurea for several weeks. This hydroxyurea induction of enzyme activity was both time-dependent and drug-concentration dependent. Immunoprecipitation experiments

performed with rabbit antiserum against the M1 subunit indicated that there was an overproduction of M1 protein in LHF cells. Evidence obtained from M2 titration experiments performed with highly purified M1 protein indicated a very large increase in M2 protein activity in LHF cells. This increase in M2 activity was also drug-dependent and was as high as 100-fold elevated in LHF cells previously cultured in the presence of 5 mM hydroxyurea for several weeks. Studies with cycloheximide and actinomycin D showed that the hydroxyurea-dependent increase in activity needed de novo protein synthesis and transcriptional activity. The effects of gossypol on the activity of ribonucleotide reductase was also investigated. Gossypol is a polyhydroxylated binapthalene compound, which has been shown to have antifertility effects on males, and antiproliferative activity on cultured mammalian cells. In this study it was demonstrated that gossypol potently inhibited the reduction CDP and ADP by ribonucleotide reductase in a competitive fashion. This work indicated that ribonudleotide reductase was an important site of action for gossypol, providing at least a partial explanation for the anti-proliferative properties of this compound.

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ABBREVIATIONS

ADP	Adenosine diphosphate
CDP	Cytidine diphosphate
GDP	Guanosine diphosphate
UDP	Uridine diphosphate
dATP	2'-Deoxyadenosine triphosphate
dCTP	2'-Deoxycytidine triphosphate
dGTP	2'-Deoxyguanosine trisphosphate
dTTP	Thymidine triphosphate
dNTP	2'-Deoxynucleoside triphosphate
dAC	5'-Deoxyadenosylcobalamin
EPR	Electron paramagnetic resonance
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
RDPR	Ribonucleoside diphosphate reductase
RTPR	Ribonucleoside triphosphate reductase
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
Tris	Tris (hydroxymethyl) aminomethane
DMSO	Dimethyl sulfoxide
mA	Milliamperes
IMPY	Pyrazolo-imidazole

INTRODUCTION

Discovery of Ribonucleotide Reductase

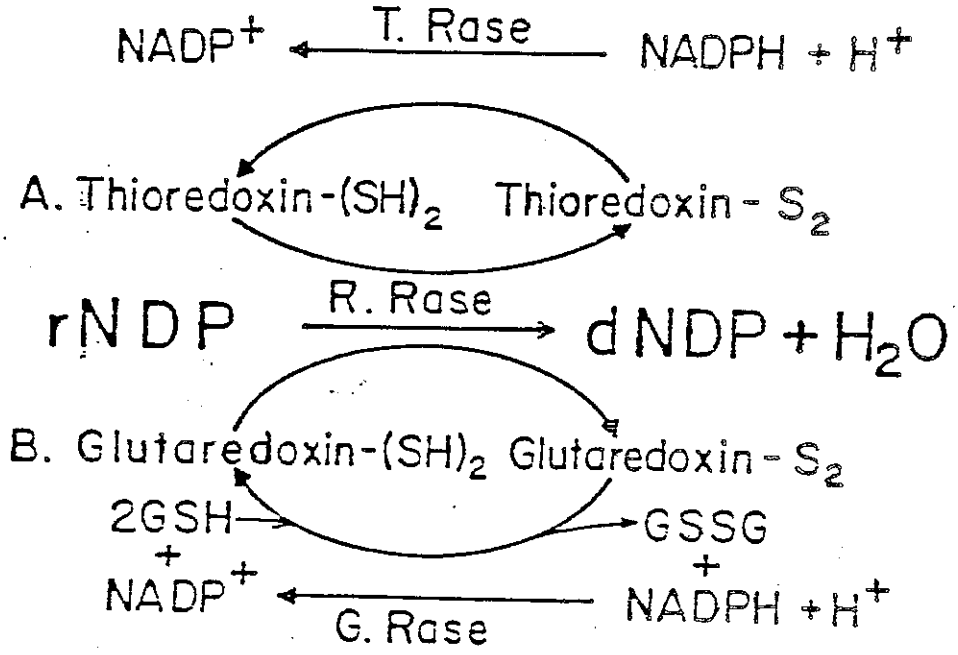
DNA synthesis requires a continuous and balanced supply of the four deoxyribonucleotides, dCTP, dTTP, dGTP and dATP. During the 1950's there were two different views concerning a possible mechanism for the synthesis of these deoxyribonucleotides (e.g. Racker, 1952; Rose and Schweigert, 1953). The first view suggested that pyrimidine and purine deoxyribonucleotides were formed de novo by a multistep pathway. This notion gained support from the detection of an aldolase activity in E. coli capable of condensing glyceraldehyde-3-phosphate and acetaldehyde to form 2'-deoxyribose tetraphosphate (Racker, 1952). However, it soon was apparent that this enzyme activity worked primarily in a degradative manner, because the enzyme was shown to have a low affinity for glyceraldehyde-3-phosphate. It was concluded that this enzyme was probably involved in degrading nucleotides. The second view proposed that deoxyribonucleotides were formed through direct reduction at the 2' position of the corresponding ribonucleotides. Early attempts to detect such a "ribonucleotide reductase" activity were not successful. However a very important observation was made in 1953 by Rose and Schweigert. They showed that uniformly labelled ^{14}C-cytidine could be incorporated into rat liver DNA, and the specific activity between the base and the sugar remained constant. Therefore, cytidine was converted to deoxycytidine without cleavage of the N-glycosidic bond. Later, evidence was obtained by other laboratories to demonstrate in a convincing manner, that ribonucleotides could be directly reduced in cell free extracts of

bacterial cells (Grossman and Hawkins, 1957; Reichard and Rutberg, 1960), chick embryo (Reichard, 1958) and mammalian cells (Moore and Hurlbert, 1960; Abrams et al, 1960). By the early 1960's it was firmly established that deoxyribonucleotides were formed by direct reduction of the corresponding ribonucleotides.

Comparison of Different Types of Ribonucleotide Reductases

Although ribonucleotide reductase is the enzyme solely responsible for conversion of ribonucleotides to deoxyribonucleotides required for DNA synthesis, two other small proteins can also participate in the reaction as hydrogen carriers (Moore et al 1964; Luthman et al, 1979) (Figure 1). These proteins are thioredoxin which functions through the thioredoxin reductase system, and glutaredoxin via glutathione and glutathione reductase (Thelander and Reichard, 1979; Wright, 1983, 1987). Studies based upon substrate specificity have led to the identification of two general types of ribonucleotide reductase. The first type reduces ribonucleoside diphosphates (EC 1.17.4.1), contains non-haem iron and a tyrosyl free radical, and is represented by the E. coli and mammalian enzymes (Thelander and Reichard, 1979; Wright, 1983, 1987). The second type reduces ribonucleoside triphosphates (EC 1.17.4.2), has an absolute requirement for 5' deoxyadenosylcobalamin (dAC) as a cofactor, and is represented by the Lactobacillus leichmanii enzyme (Singh et al, 1977; Thelander and Reichard, 1979). Interestingly, Bacillus megaterium, Corynebacterium nephridii and Rhizobium melilote exhibit enzyme activity intermediate between these two classes of ribonucleotide reductase. Enzymes from these organisms reduce

Figure 1. Reduction of ribonucleotides (rNDP) to deoxyribonucleotides (dNDP) by ribonucleotides reductase (R. Rase) (Wright, 1983, 1987). Two small protein molecules, thioredoxin and glutaredoxin serve as intermediate hydrogen carriers; thioredoxin through the thioredoxin reductase (T. Rase) system (A) and glutaredoxin (GSH) via the glutathione and glutathione reductase (G. Rase) system (B). The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) provides the ultimate reduction potential for both systems.

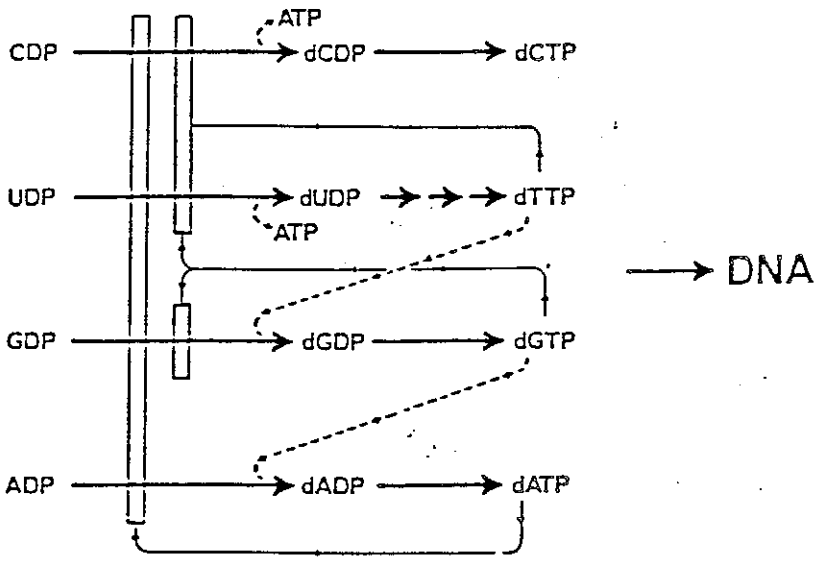

NADP+
T. Rase
NADPH + H+
A. Thioredoxin-(SH)2
Thioredoxin - S2
rNDP
R. Rase
dNDP + H2O
B. Glutaredoxin-(SH)2
Glutaredoxin - S2
2GSH
GSSG
+
NADP+
+
NADPH + H+
G. Rase

ribonucleoside diphosphates but require 5' deoxyadenosylcobalamin for activity (Yau and Wachsman, 1973; Crowles and Evans, 1968; Tsai and Hogenkamp, 1980).

Allosteric Regulation of Mammalian Ribonucleotide Reductase

In keeping with the importance of ribonucleotide reductase activity in the physiology of the cell, the substrate specificity and activity of the mammalian enzyme is regulated in a complex fashion by nucleoside triphosphate effectors (Figure 2) (Thelander and Reichard, 1979; Wright, 1983, 1987). For example, the reduction of CDP to dCDP and UDP to dUDP occurs in the presence of ATP. The reduction of GDP to dGDP requires activation by dTTP, and the reduction of ADP to dADP requires the presence of dGTP. Reduction of all four ribonucleotide substrates is inhibited in the presence of dATP. The allosteric scheme for mammalian ribonucleotide reductase suggests that deoxyribonucleoside diphosphate formation begins with a reduction of CDP and UDP by an ATP activated enzyme, proceeds to GDP reduction via a dTTP regulated activity, and eventually reaches ADP reduction by a dGTP activated activity. Accumulation of dATP during the slowing down of DNA synthesis completely inhibits ribonucleotide reduction, because dATP is a potent inhibitor of all four ribonucleotide reductions. In addition to the above, dTTP is a good inhibitor of pyrimidine reductions and dGTP is a negative feedback effector for GDP reduction and inhibits pyrimidine reductions. Some studies show that intracellular regulation of mammalian ribonucleotide reductase may be even more complex than briefly described above (e.g. Fox, 1985; Hards and Wright, 1984), but, this general model for the

Figure 2. Schematic representation of the allosteric regulation of deoxyribonucleotide synthesis. The broken arrows stand for positive effects, the open bars for negative effects. This scheme was constructed from studies with calf thymus enzyme preparations (Eriksson et al 1979). Note that ATP exhibits positive effect on the reduction of all four ribonucleotide substrates and is left out of the scheme for simplicity.


ATP
CDP
dCDP
dCTP
UDP
dUDP
dTTP
ATP
DNA
GDP
dGDP
dGTP
ADP
dADP
dATP

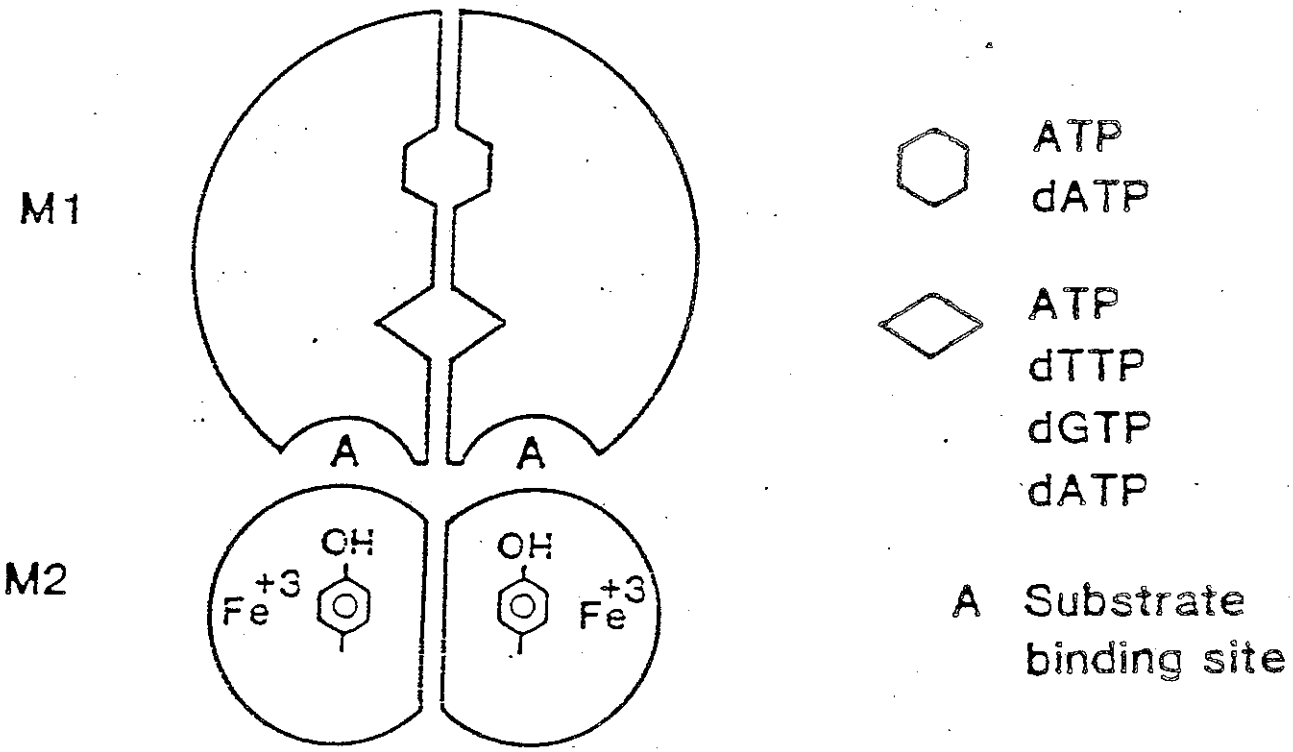
regulation of ribonucleotide reductase has been very useful in investigations of this activity in a variety of mammalian cells (Wright, 1987).

Structure of Mammalian Ribonucleotide Reductase

Mammalian ribonucleotide reductase can be separated into two components (Moore, 1977; Cory et al , 1978; Chang and Cheng, 1979), which are frequently referred to as M1 and M2 (Figure 3), similar to the B1 and B2 subunits described earlier for the bacterial reductase (Thelander et al, 1980; McClarty et al, 1986a). Substrates and effectors bind to protein M1, which is a dimer of molecular weight 170,000 (Thelander et al, 1980; Eriksson et al, 1982). The M2 protein is also a dimer, with a molecular weight of 88,000 (Thelander et al, 1985; McClarty et al, 1987a), and contains the non-haem iron and tyrosyl free radical critically required for reduction of the ribonucleotides. The presence of a unique tyrosyl free radical as part of the functional M2 component allows the determination of M2 protein expression in whole cells by measuring, with the use of electron paramagnetic resonance spectroscopy, the characteristic asymmetric doublet of the tyrosyl free radical (Graslund et al, 1982; Wright et al, 1987a,b; McClarty et al, 1987a).

Enzyme activity depends upon the presence of both M1 and M2 components (Cory et al, 1978; McClarty et al, 1986a). However, different mechanisms control the levels of the two proteins during cell growth (Eriksson and Martin, 1981; Cory and Fleischer, 1982; Eriksson et al, 1984; Engstrom et al, 1985; Wright et al, 1987a,b; McClarty et al, 1987a; Choy et al, 1988). These regulatory

Figure 3. A subunit structure model for ribonucleotide reductase (Wright, 1983; 1987). There are two independent regulatory domains on the M1 protein dimer. One of these (⬡) is responsible for regulating overall activity through binding of ATP (activator) or dATP (inhibitor). The other domain regulates substrate specificity through the binding of ATP, dTTP and dGTP. Apparently, dATP can also bind to the substrate specificity site of M1 and mimic the effects observed with ATP. Protein M2 also forms a dimer containing non-heme iron which is involved in stabilizing the tyrosine free radical.


M1
M2
A
A
OH
OH
Fe+3
Fe+3
ATP
dATP
ATP
dTTP
dGTP
dATP
A Substrate binding site

differences are unlike the observations obtained with E. coli, where the two equivalent genes (B1 and B2) are located in one operon, and their synthesis is coordinately regulated (Hanke and Fuchs, 1983; Carlson et al, 1984). Also, in contrast to the E. coli findings, the rodent and human M1 and M2 genes have recently been mapped to different chromosomes (Yang-Feng et al, 1987; Tonin et al, 1987) and there appear to be pseudogenes for M2 in mammalian cells (Yang-Feng et al, 1987; Wright et al, 1987b).

Biological Significance of Ribonucleotide Reductase

Since ribonucleotide reductase performs such a critical function in DNA synthesis, and therefore, cell division, it is often the subject of intensive research efforts aimed at gaining a better understanding of the regulation of DNA synthesis and cell proliferation (Thelander and Reichard, 1979; Fox, 1985; Wright, 1987). A thorough understanding of the structural and regulatory characteristics of ribonucleotide reductase should substantially improve our comprehension of the overall regulation of DNA synthesis and cell division. Alterations in ribonucleotide reduction have been correlated with important changes in the biological properties of mammalian cells (Wright, 1983, 1987). For example, it has been observed that neoplastic cells may exhibit a higher ribonucleotide reductase activity than normal cells with similar growth rates (Takeda and Weber, 1981; Weber, 1983; Tagger and Wright, 1984). It has also been suggested that defective purine nucleoside phosphorylase activity or adenosine deaminase activity could upset the delicate balance of intracellular deoxyribonucleotide

concentrations, and interfere with the normal regulation of ribonucleotide reductase through its allosteric properties, leading to certain immunodeficiency diseases in humans (Ullman et al, 1976; Chan, 1978; William et al, 1987). Also, the critical position of ribonucleotide reductase in DNA synthesis is emphasized by the observations that alterations in this enzyme activity can lead to increased rates of spontaneous mutation due to an imbalance of endogenous deoxyribonucleotide pools (Weinberg et al, 1981; Chan et al, 1981; Arpaia et al, (1983). However, this relationship between altered deoxyribonucleotide pools and increased spontaneous mutation rates is not always observed (Tagger and Wright, 1986). In addition to the above, alterations in ribonucleotide reductase have been implicated in modifications to the replicative abilities of human fibroblasts (Dick and Wright, 1984), and the differentiation properties of rat myoblasts (Creasey and Wright, 1984).

The unusual structural, regulatory, and biological properties of the mammalian ribonucleotide reductase make it an important enzyme to investigate in detail. To carry out such a study this laboratory has initiated a biochemical genetic approach to the problem (Wright and Lewis, 1974; Wright et al, 1987b; Wright, 1983, 1987; McClarty et al, 1986a, 1987a,b; Choy et al, 1988). Cytotoxic drugs, whose intracellular target is ribonucleotide reductase, have been used as selective agents in culture to isolate drug resistant cell lines with specific modifications in enzyme activity. The antitumor agent, hydroxyurea (Bolin et al, 1982; Engstrom, P.F., et al, 1984), is particularily useful for these types of studies. This drug enters

mammalian cells by a diffusion process (Morgan et al, 1986; Tagger et al, 1987), and specifically inhibits DNA synthesis and therefore cell division, by destroying the M2 tyrosyl free radical of ribonucleotide reductase (Graslund et al, 1982; McClarty et al, 1987a). The characteristics of a mouse cell line selected for resistance to cytoxic effects of hydroxyurea are described in this thesis and elsewhere (McClarty et al, 1986a,b, 1987a; Choy et al, 1988).

Chemical Mechanism of Ribonucleotide Reduction

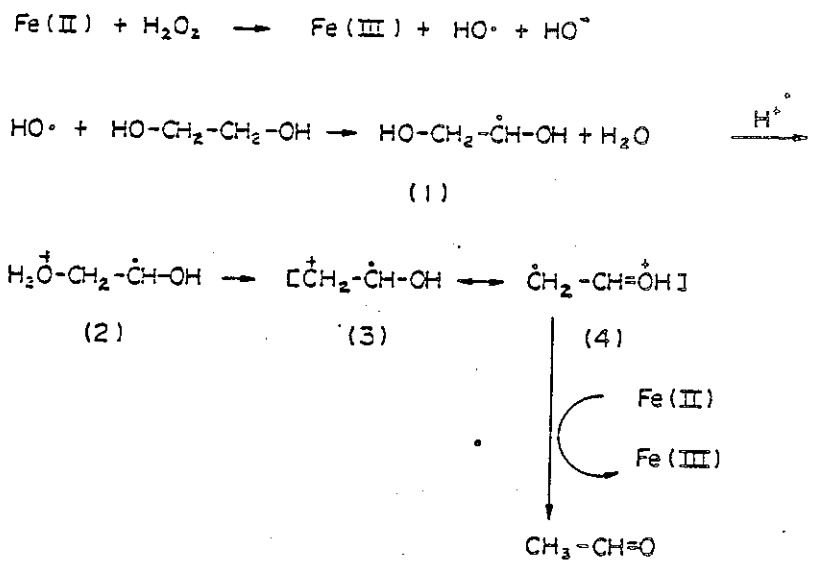
For many years after the discovery of the enzyme ribonucleotide reductase, the actual chemical mechanism of ribonucleotide reduction remained unclear and no satisfactory model for the reaction was proposed. The discovery that a stable tyrosyl free radical is present in the ribonucleoside diphosphate reductase prompted the consideration of a mechanism involving free radical in the reduction of ribonucleotides (Ehrenberg and Reichard, 1972). In order to be consistent with existing evidence, any hypothesis must satisfy the following two criteria: (1) the hydroxyl (OH) group at the 2' position of the ribose is replaced sterospecifically by hydrogen, and other parts of the ribose moiety must remain unchanged. (2) the tyrosyl free radical in the small subunit of the reductase (M2 or B2) is not lost during the reaction. A chemical mechanism was proposed for the ribonucleotide reductase that contain a tyrosyl free radical (Reichard and Ehrenberg, 1983; Ashley and Stubbe, 1985) as well as for the 5' deoxyadenosylcobalamin (dAC)-dependent ribonucleotide reductase (Ashley and Stubbe, 1985). The reaction can be divided into two main chemical events. One of the necessary chemical events

is the cleavage of the 2' carbon-hydroxyl bond through the generation of a radical cationic intermediate. The second step involves the reduction of the radical cation by the redox reactive dithiol.

The proposed model for the mechanism of 2' carbon-hydroxyl bond cleavage is initiated by Fenton's Reagent [Fe(II)/H_2O_2] as shown in Figure 4 (Ashley and Stubbe, 1985). This hypothesis is based on kinetic studies (Walling and Johnson, 1975) and electron paramagnetic resonance (EPR) studies (Gilbert et al, 1972; Buley et al, 1966). This model system suggested that hydrogen peroxide (H_2O_2) was initially reduced by Fe(II) to generate the reactive hydroxyl radical. This hydroxyl radical was capable of abstracting a hydrogen from the substrate to produce a hydroxyalkyl radical (1) which would undergo protonation to form (2); H_2O was then lost to give a transient radical cation (3). This radical cation (3) is a resonance form of the conjugated aldehyde radical (4), and a further one electron reduction by Fe(II) and tautomerization yielded the product aldehyde. Supporting evidence for this mechanism was provided by the detection of the EPR spectrum of (1) at pH 7, but only the spectrum of the aldehyde radical (4) was detected at about pH 0. This suggested that a protonation step was involved in converting (1) to (4).

This model provided a pathway for the cleavage of the 2' carbon-hydroxyl bond but it did not fully explain the intermolecular reduction observed in ribonucleotide reductase catalysis. It is believed that in the actual reductase catalysis, the radical cation intermediate undergoes a 2 electron ($2e^-$) reduction performed by the

Figure 4. Fenton's Reagent: a model system for the cleavage of carbon-oxygen bond (Ashley and Stubbe, 1985).


Fe(II) + H2O2 → Fe(III) + HO· + HO⁻
HO· + HO-CH2-CH2-OH → HO-CH2-ĊH-OH + H2O
H+
(1)
H2Ö-CH2-ĊH-OH → [ĊH2-ĊH-OH ⟷ ĊH2-CH=ÖH]
(2)
(3)
(4)
Fe(II)
Fe(III)
CH3-CH=O

dithiol group in the enzyme instead of an one electron ($1e^-$) reduction by Fe(II) of Fenton's Reagent. This 2 electron reduction (hydride reduction) of the radical cation and the conjugated aldehyde radical would generate a deoxygenated hydroxylalkyl radical. These two reductive reactions are compared in Figure 5.

Following the model systems for the two basic reactions, a mechanism for ribonucleotide reductase catalysis was proposed for the enzyme that contain a tyrosyl free radical (Reichard and Ehrenberg, 1983; Ashley and Stubbe, 1985). The reaction mechanism illustrated in Figure 6 assumed the presence of a radical group Enz-$X^{\bullet}$ and a dithiol group at the active site. Catalysis begins with the abstraction of the 3' hydrogen atom by Enz-$X^{\bullet}$. The 2'-hydroxyl group can be protonated by perhaps one of the active site thiols and loss of H_2O would produce a radical cation. This intermediate would undergo hydride reduction by the dithiol to generate the product radical which could abstract the hydrogen atom from Enz-XH to form product and regenerate the active site radical.

Several predictions specified by this reaction scheme have been experimentally tested. This reaction mechanism predicted that the 3' carbon-hydrogen bond had to be broken during substrate reduction. The possibility was investigated by using [3'-^{3}H]ADP and [3'-^{3}H]UDP as the diphosphate substrates for ribonucleoside diphosphate reductase (RDPR) (Ashley et al, 1986). It was found that a small quantity of ^3H_2O was released into the solvent during the reduction. This indicated that the hydrogen on the 3'-carbon was transiently located in an exchangeable position during the reduction (Stubbe et

Figure 5. A scheme for hydride reduction of the radical cation intermediate by dithiol group of ribonucleotide reductase (Ashley and Stubbe, 1985).

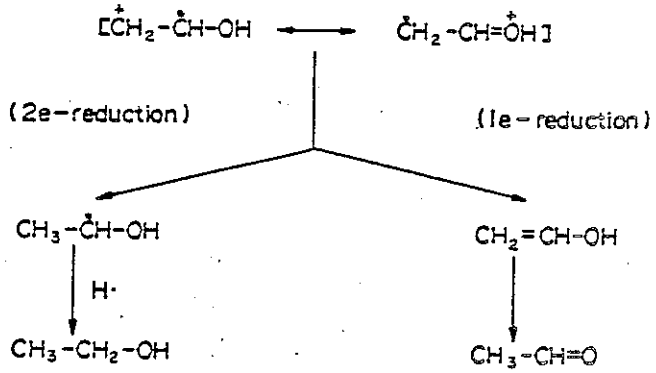
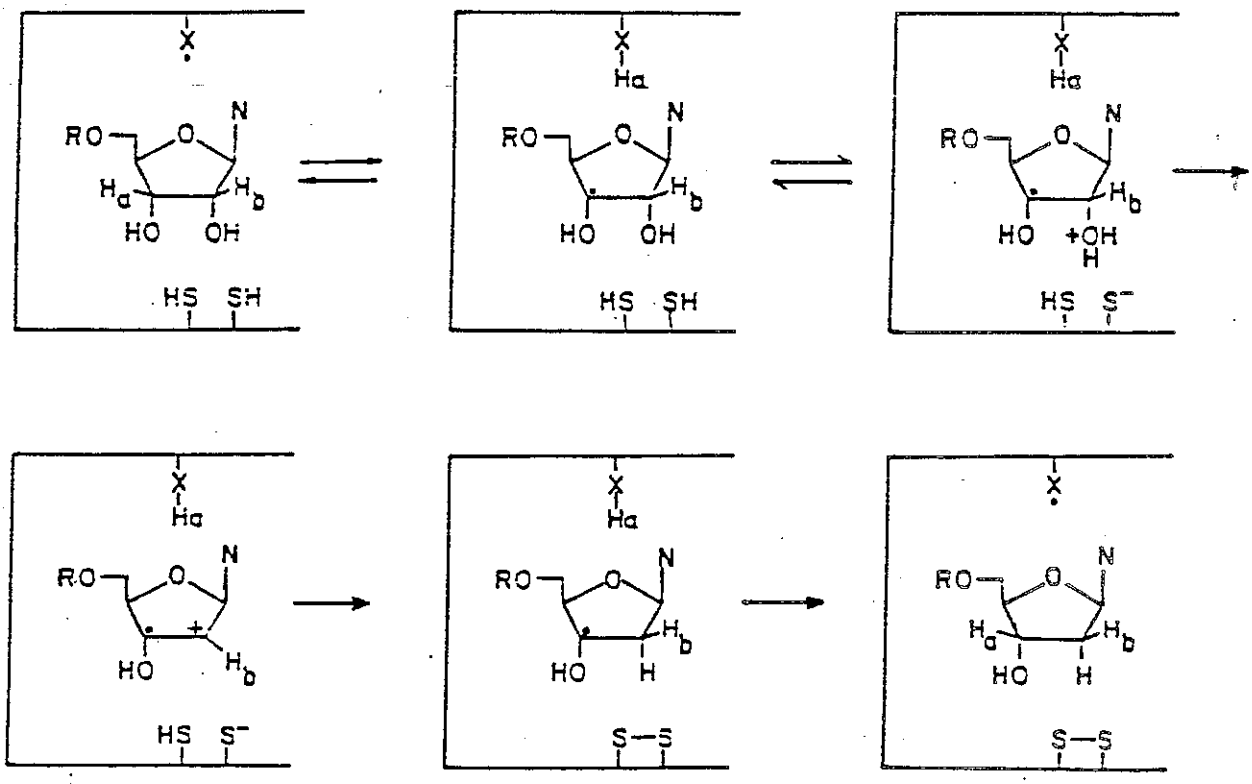

[CH2-CH-OH ⟷ CH2-CH=OH]
(2e−reduction)
(1e − reduction)
CH3-CH-OH
H·
CH3-CH2-OH
CH2=CH-OH
CH3-CH=O

Figure 6. A model for the chemical mechanism of ribonucleotide reductase catalysis involving a radical group Enz-X$^{\cdot}$ and a dithiol at the active site (Ashley and Stubbe, 1985).

X= protein



al, 1983).

The proposed scheme also suggested that after the cleavage of the 3' carbon-hydrogen bond, the same hydrogen is returned to the original position at the end of the reaction. This was tested by using [3'-^{2}H]UDP as the substrate for RDPR (Stubbe et al, 1983). The product of the reduction was isolated and examined with nuclear magnetic resonance spectroscopy (NMR). It was observed that the 3'-position was completely deuterated within the detectable limits. The evidence from these two studies indicated that the hydrogen on the 3'-carbon was initially removed and was replaced at the end of the reaction. These findings were in agreement with the predictions of the proposed reaction mechanism.

The involvement of the tyrosyl free radical in the reaction must also be established. It has been shown that the B2 subunit of E. coli exhibits a characteristic optical absorbance at 410 nm only when the tyrosyl free radical is present (Sjoberg et al, 1978; Sealy et al, 1985). Therefore, the abstraction of the 3' hydrogen atom by the tyrosyl radical should produce a transient loss of the characteristic absorbance. Attempts to demonstrate this change have been unsuccessful (Ashley and Stubbe, 1985). This negative evidence appears to suggest that the tyrosyl radical has no direct role in the catalysis. However, several explanations should be considered: firstly, the concentration of the reduced tyrosine species may be undetectably low because the rate of hydrogen abstraction by the tyrosyl radical is much lower than the rate at which the hydrogen is returned to the 3'-carbon position. Alternatively, the hydrogen

abstraction and return to the product may reach steady-state within the dead time of the stopped-flow spectrophotometer. Both explanations seemed chemically reasonable and the involvement of tyrosyl radical in the reduction reaction is still a likely possibility.

Ashley and Stubbe (1985) have also proposed a chemical mechanism for 5' deoxyadenosylcobalamin (dAC)-dependent ribonucleoside triphosphate reductase (RTPR). Although the dAC-dependent reductases do not naturally contain a radical group, the mechanism of catalysis is believed to involve free radical. The radical species is generated upon homolysis of the carbon-cobalt bond by RTPR and the resulting products are Cob(II)alamin and the 5' deoxyadenosyl radical (Babior and Krouwer, 1979). The reaction catalyzed by the two classes of ribonucleotide reductases showed many similar characteristics. (1) In both reactions, the 2'-hydroxyl group is replaced by a hydrogen that is ultimately derived from the solvent, and the original sterochemistry is maintained about the 2'-carbon. (2) Furthermore, both enzymes couple substrate reduction to oxidation of a dithiol, which must subsequently be reduced by an exogenous system. (3) Both classes of enzymes are inactivated by substrate analogs such as 2'-deoxy-2'-halonucleotides and 2'-azido-2'-deoxynucleotides (Thelander et al, 1976; Harris et al, 1984). (4) Finally, both reactions are believed to involve free radicals; a stable tyrosyl radical for the class I enzyme and 5'-deoxyadenosyl radical for the class II enzyme. This evidence strongly suggested that the mechanism of catalysis of the two classes

of reductase are very similar despite the significant differences in structure and cofactor requirements.

MATERIALS AND METHODS

Cell Lines and Culture Conditions.

Wild type mouse L cells: The mouse L cells used in this study were originally isolated by Earle (1943). This immortal mouse fibroblast cell line has been studied extensively and used successfully to isolate a variety of mutant phenotypes (e.g. Dubbs and Kit, 1964; Thompson et al, 1970, 1971; Kuzik and Wright, 1980; Wright et al 1980; McClarty et al, 1986a, 1987a). The mouse L cells grow well in culture and exhibit a doubling time of about 18 hours. Cells were cultured on the surface of plastic cell culture plates (Lux Scientific Ltd.) with Alpha modified minimal essential medium (αMEM) (Flow Laboratories Ltd.) supplemented with penicillin G (100 units/ml) (Sigma Chemicals), streptomycin sulfate (100 μg/ml) (Sigma Chemicals) and 10% (v/v) fetal bovine serum (FBS) (Gibco, Ltd.). Cells were incubated at 37°C in a humidity-controlled incubator containing a 5% carbon dioxide atmosphere.

Hydroxyurea resistant mouse L cell lines: A highly hydroxyurea resistant cell line, designated as LHF, was selected from the wild type population by culturing the wild type cells in stepwise increasing concentrations of hydroxyurea as follows: Wild type cells -> 0.35 mM -> 1.3 mM -> 1.5 mM -> 2.0 mM -> 3.0 mM -> 4.0 mM -> 5.0 mM hydroxyurea (LHF cell line) (McClarty et al, 1986a). The LHF cells were continuously cultured in the presence of 5 mM hydroxyurea in MEM containing 10% FBS. Some LHF cells were also grown in the absence of hydroxyurea for specific experimental purposes. For the sake of clarity, the cells that were cultured in the presence of hydroxyurea will be denoted as LHF^+ and cells that were grown in the absence of

hydroxyurea for at least 2 weeks will be referred to as LHF^- cells.

Routine Cell Culture Procedures.

Trypsin treatment of cultured cells: To enzymatically detach cells from the surface of tissue culture plates, the medium was first removed by aspiration and the plates were washed once with 5 ml of phosphate buffered saline (PBS), pH 7.3. A 2 ml aliquot of a 0.2% trypsin (Bacto trypsin, Difco) in sterile phosphate buffered saline (PBS) solution was added to the culture plates. The trypsin treatment was carried out at room temperature and usually required 3-5 minutes. When the cells appeared detached, 3 ml of αMEM containing 10% FBS was added. The cells were transferred into a sterile centrifuge tube and recovered by centrifugation at 500 x g for 5 minutes and then resuspended in an appropriate medium.

Subculture: Cell cultures that approach confluence were subcultured. The cells were detached from the surface with the aid of trypsin solution and washed in αMEM containing 10% FBS. The density of the cell suspensions was determined by the use of an electronic particle counter (Coulter Electronics, Model A). An aliquot of 1 x 10^5 cells was transferred to a fresh 100 mm tissue culture plate containing 10 ml of αMEM containing 10% FBS.

Cold storage and resuscitation of frozen cells: For long term cold storage, a population of cells was resuspended in αMEM containing 10% FBS and 10% dimethyl sulfoxide, (DMSO) (Fisher Scientific) at a density of about 5 x 10^6 cells per ml. Cells were frozen in 1 ml aliquots in a cryotube (Nunc) at -70°C. For reculturing of frozen cells, the content of the cryotube was thawed quickly in a 37°C

waterbath. The cells were washed once with MEM containing 10% FBS and then resuspended in the same medium for incubation in a culture plate.

Determination of Protein Concentration

The concentration of protein in cell free preparations was measured using a Bio Rad protein assay kit (Bio Rad Laboratories), Technical bulletin 1051. Protein concentrations were determined by using the Standard Assay Procedure described in the instruction manual. Purified bovine serum albumin (Sigma Chemical Co.) was used to generate a protein standard curve covering the range of zero to 80 µg of protein.

Determination of Growth Rates

Exponentially growing cells were removed from culture plates with trypsin solution. Cells were washed with PBS and counted with the aid of an electronic particle counter (Coulter Electronics). A set of culture plates (60 mm diameter) containing 5 ml of MEM containing 10% FBS was inoculated with 1×10^5 cells. After an overnight incubation at 37°C, cells from two of the plates were independently harvested with trypsin solution and the total number of cells on each plate was determined. These cell numbers were considered as the number of cells at time zero. This will ensure that the cells are in log phase of growth and minimizes the distortion caused by a lag period in the beginning. At every predetermined time interval, another two culture plates were harvested and the total number of cells on the plates were determined.

Determination of Colony-forming Ability in the Presence of Hydroxyurea

To determine the colony forming ability, exponentially growing cells were harvested with the aid of trypsin solution and counted as described above. A pre-determined number of cells ranging from 100 to 1000 were added to 100 mm culture plates with 10 ml of αMEM plus 10% FBS. After an incubation period of about 8 to 10 days at 37°C, the cells were stained with a filtered 50% solution of ethanol saturated with methylene blue (Sigma Chemical Co.) at room temperature for about 15 minutes. Colonies consisting of more than 40 cells were counted. Plating efficiency was calculated by dividing the number of cells added into the number of colonies formed on the plate. The plating efficiency of the cell lines used in the study varied between 0.70 and 0.90.

The effect of various drugs on the growth of cells can be studied by determining the relative plating efficiency (RPE). The RPE is defined as the plating efficiency in the presence of drug divided by the plating efficiency in the absence of drug. The RPE was determined by plating a pre-determined number of cells in culture plates containing medium with increasing concentrations of drug, and in the absence of drug as a control. This method was used to study the effect of hydroxyurea on the colony forming ability of wild type mouse L cells and drug resistant mutant cells (LHF).

Assay of Ribonucleotide Reductase Activity in Permeabilized Cells

Permeabilization of cells: Intracellular ribonucleotide reductase activity of mouse L cells was measured by making the cells permeable to ribonucleotides (Lewis et al, 1978; Hards and Wright, 1983; Wright et al, 1981). Exponentially growing wild type cells were

plated at a density of 3 x 10^6 cells per 150 mm tissue culture plate containing αMEM plus 10% FBS with or without 5 mM hydroxyurea, and incubated at 37°C for 40 hours. Usually, a cell density of about 8 ± 0.8 x 10^6 cells per plate was achieved. All plates were at a subconfluent stage. Cells were removed with the aid of 0.2% trypsin in PBS solution, centrifuged, washed with PBS and resuspended at 1 x 10^7 cells/ml of permeabilizing buffer. This buffer contained: 50 mM N-2-hydroxyethylpiperazine-N'2-ethane-sulfonic acid (Hepes) (Sigma Chemical Co.), 1% tween 80 (J.T. Baker), 0.25 M sucrose (Fisher Scientific) and 2 mM dithiothreitol (DTT) (Boehringer Mannheim), pH 7.2. The cells were incubated at room temperature (22°C) for 30 minutes with constant stirring by using a small magnetic stir bar (2x7 mm). The cells were then centrifuged (500 x g for 10 minutes) and resuspended with permeabilizing buffer at 1.5 x 10^7 cells/ml. 200 µl (3 x 10^6 cells) of this cell suspension was used in each assay point.

Reduction of Cytidine Diphosphate (CDP): To measure CDP reductase activity, 200 ul of permeabilized cell suspension which contained 3 x 10^6 cells was added to 100 µl of assay mixture. The resulting reaction mixture contained 3 x 10^6 permeabilized cells, 50 mM Hepes pH 7.2 (Hards and Wright, 1981), 2 mM ATP (Sigma Chemical Co.), 6 mM DTT, 8 mM $MgCl_2$ (Fisher Scientific), 0.4 mM ^{14}C-CDP (5,000 dpm/nmole) (Amersham Ltd.), 0.67% tween 80 and 0.167 M sucrose. The reaction mixture was incubated for 20 minutes at 37°C with constant stirring. The reaction was terminated by heating in a boiling water bath for 4 minutes. As a control for these experiments, the reaction mixture was boiled immediately upon addition of permeabilized cells

and then incubated at 37°C for 20 minutes. Nucleotides were converted to nucleosides by addition of 2 mg Crotalus atrox venom (Sigma) with 10 mM $MgCl_2$ in 0.1 M Hepes buffer, pH 8.0 at 37°C for 2 hours. After the reactions were terminated by boiling for 4 minutes, 0.5 ml of deionized water was added to each reaction tube and precipitated material was removed by centrifugation. The newly formed deoxycytidine was separated and measured by the method of Steeper and Steuart (1970). The supernatant was passed through a Dowex-1-borate column 5 x 80 mm (Bio Rad Laboratories). The deoxycytidine was eluted with 5 ml of deionized water and the eluate was mixed with 10 ml of Scintiverse II (Fisher Scientific) and counted with a liquid scintillation counter (Beckman, LS9800). Enzyme activity was expressed as nmoles of dCDP formed/3 x 10^6 cells/hour.

Reduction of adenosine diphosphate (ADP): The procedure for assaying ADP reductase activity in permeabilized cells was similar to the method for the CDP reductase assay except for differences in the composition of the permeabilizing buffer, the reaction mixture and the procedure in separating deoxyadenosine from adenosine. The permeabilizing buffer for the ADP reductase assay contained: 1% Tween 80, 0.25 M sucrose 2 mM DTT, 20 mM piperzine-N,N'-bis-(2-ethane sulfonic acid) (Pipes) (Sigma) pH 6.8. The reaction mixture contained the following: 0.5 mM dGTP (Sigma), 6 mM DTT, 0.4 mM ^{14}C-ADP (5000 dpm/nmol), 0.67% Tween 80, 0.167 M sucrose in 50 mM Pipes, pH 6.8 and 3 x 10^6 permeabilized cells (Hards and Wright, 1981).

Deoxyadenosine and adenosine were separated on a 5 x 80 mm column of Dowex-1-borate resin (Bio Rad). The column was equilibrated with 8

ml of 1 mM sodium borate solution prior to loading the samples. The deoxyadenosine was eluted with 20 ml of 1 mM sodium borate. The first 4 ml of eluant was discarded and the following 16 ml was collected. A 4 ml aliquot of the collected eluant was mixed with 10 ml of Scintiverse II (Fisher Scientific) and radioactivity was measured with a liquid scintillation counter (Beckman, LS9800).

Assay of Ribonucleotide Reductase Activity in Cell-free Enzyme Preparations.

Ribonucleotide reductase activity in mouse L cells was also measured in cell-free preparations using the method described by Cory et al (1973). Exponentially growing wild type and drug resistant mutant populations were harvested and washed twice with PBS, pH 7.2. The cells were resuspended at approximately 8 x 10^6 cells per 200 ul of 1 mM DTT in 20 mM Tris-HCl pH 7.2. The cell suspension was disrupted with 10 second pulses of sonication 3 times at 30% power, and then centrifuged at about 12,000 x g (Micro Centaur, MSA) at 4°C for 10 minutes to remove cellular debris. An aliquot of the supernatant containing approximately 200 µg of protein was added to 25 µl of reaction mixture. The final volume of the reaction was made up to 150 µl with buffer containing 1 mM DTT, 20 mM Tris-HCl pH 7.2. The final concentrations of the essential reagents contained in the reaction were: 4 mM magnesium acetate, 6 mM DTT, 1 mM ATP and 50 M ^{14}C-CDP (3,000 dpm/nmole).

After incubating at 37°C for 20 minutes, the reactions were boiled for 4 minutes, followed by the addition of 50 µl (1 mg) of Crotalus atox venon with 10 mM $MgCl_2$ in 0.1 M Hepes pH 8.0. After a

90 minute incubation, the reaction was stopped by boiling for about 4 minutes. The quantitation of the reaction product deoxycytosine was performed as described for the CDP reductase assay in permeabilized cells.

Preparation of Dowex-1-borate resin

The ion exchange resin was purchased from Bio Rad Laboratories Ltd. as Dowex-1-chloride, 200-400 mesh and the chloride ions were replaced with borate ions. About 450 g of resin was resuspended in 4 litres of saturated sodium borate solution and stirred overnight at room temperature. The resin was collected by filtration and then resuspended in another 4 litres of saturated sodium borate solution. After overnight stirring, the resin was again collected by filtration and washed with 16 litres of deionized water. Finally, the Dowex-1-borate resin was resuspended in about 500 ml of water to make a thick slurry and stored at 4°C.

Purification of M1 protein from lamb thymus

Homogenization: The purification was carried out according to the method of Engstrom et al (1979), Thelander et al (1980) and Mattaliano et al (1981). Thymus of young lambs were supplied by a local slaughter house (Ba Ba Black Sheep, Winnipeg). For each preparation, about 2 kg of frozen thymus were homogenized with a polytron (Sybron-Brinkman) in 20 mM Tris-HCl pH 7.6 then centrifuged at 17,000 x g (JA-10 rotor, Beckman) for 20 minutes to remove insoluble materials. The supernatant was filtered through glass wool to remove lipid material.

Precipitation with Streptomycin Sulfate: Streptomycin sulfate

powder (Sigma) was added to the glass wool filtered supernatant to make a 0.65% solution. This mixture was stirred for 30 minutes at 4°C and then centrifuged at 17,000 x g for 20 minutes and the precipitate was discarded.

Precipitation with ammonium sulfate: Ammonium sulfate crystals (Fisher Scientific) were added gradually, with mixing at 4°C, to the supernatant to obtain a 40% saturated solution. The solution was stirred at 4°C for 30 minutes before being centrifuged at 17,000 x g for 20 minutes. The supernatant was discarded and the precipitate was dissolved in 20 mM Tris-HCl pH 7.6 buffer and dialyzed overnight against two times 22 litres of 20 mM Tris-HCl pH 7.6 buffer solution.

DEAE-cellulose chromotagraphy: After dialysis, the preparation was treated with DEAE-cellulose (Sigma) in a batchwise fashion. The DEAE-cellulose resin was prepared by alternating washes with 0.25 M hydrochloric acid (HCl) and 0.25 M sodium hydroxide (NaOH) solution repeatedly. The prepared resin was mixed with dialysate and stirred at 4°C for 30 minutes. The slurry was suction-filtered through a Whatman No. 4 filter paper. The filtrate was discarded. Proteins that were bound to the resin were eluted with stepwise increasing concentrations of potassium chloride (Fisher Scientific). First, the resin was resuspended in 0.075 M KCl, 20 mM Tris-HCl buffer, pH 7.6. After 30 minutes of stirring at 4°C, it was filtered and the filtrate was discarded. The collected resin was washed in the same buffer and then resuspended in a buffer containing 0.16 M KCl, 20 mM Tris-HCl, pH 7.6. The suspension was stirred and filtered as before and the filtrate which contained the enzyme was saved. Proteins in the

filtrate were precipitated by the gradual addition of solid ammonium sulfate with stirring at 4°C to make a 75% saturated solution. The precipitate was collected by centrifugation (17,000 x g, 20 minutes), and then dissolved in 0.1 M KCl, 1 mM DTT, 20 mM Tris-HCl buffer pH 7.6. This preparation was dialyzed overnight against 2 times 22 litres of the same buffer at 4°C.

Chromatography on dATP-sepharose: The synthesis of dATP-sepharose was carried out approximately according to the method of Knorre et al (1976). A column of 25 x 60 mm of dATP sepharose was equilibrated with 1 mM DTT, 0.1 mM KCl, 20 mM Tris-HCl, pH 7.6 at room temperature. Approximately 1/3 of the dialysate was applied to the column with a flow rate of about 20 ml per hour. The column was washed with the same buffer until absorbance at 280 nm (A_{280}) was approaching zero. Proteins with low affinity for the ligand were eluted with about 30 ml of 0.5 mM ATP, 1 mM DTT, 0.1 M KCl, 20 mM Tris-HCl pH 7.6. The column was futher eluted with the same buffer containing 50 mM ATP. The elution was carried out until the effluent absorbance at 295 nm (A_{295}) was less than 0.05 unit. The 50 mM ATP fraction was brought to 75% ammonium sulfate saturation, and the precipitate appearing after 30 minutes at 4°C was recovered by centrifugation. The precipitate was dissolved in 1 ml of 1 mM DTT, 0.1 M KCl, 20 mM Tris-HCl pH 7.6 and dialyzed for 4 hours at 4°C against the same buffer. The preparation was applied to a fresh dATP-sepharose column (1 x 5 cm) and elution was carried out as before; first with Tris-HCl buffer containing 0.5 mM ATP followed by buffer containing 50 mM ATP. The elution was monitored by measuring

A_{295} of the effluent. Both fractions were concentrated by ammonium sulfate precipitation (75% saturation) and dialyzed against 1 mM DTT, 0.1 M KCl, 20 mM Tris-HCl pH 7.6 overnight at 4°C. These fractions were stored at -70°C.

The dATP-sepharose was regenerated after each use with 6 M guanidinium-HCl solution (Fisher Scientific). About 100 ml of 6 M guanidinium-HCl was applied to the larger column and 15 ml to the smaller column, followed by extensive washing with 20 mM Tris-HCl pH 7.6 buffer.

Chromatography on DEAE cellulose in the presence of ATP and magnesium acetate: The fraction eluted with 50 mM ATP solution from the dATP-sepharose column was shown to contain two predominant proteins (86 and 58 K dalton) when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). This preparation was further purified on a DEAE-cellulose column (1 x 4 cm DE52, Whatman). The preparation was made to contain 1 mM DTT, 4 mM ATP, 4 mM magnesium acetate 0.05 M KCl in 20 mM Tris-HCl pH 7.6 and allowed to stand at 0°C for 30 minutes. It has been shown that when ATP, one of the allosteric effectors of ribonucleotide reductase, was bound to the M1 subunit, a higher concentration of KCl (0.25 M) was required for the elution of the M1 subunit from the DEAE-cellulose column (Mattaliano et al, 1981). The column was equilibrated with the same Tris-HCl solution at 4°C. The sample was applied to the column at about 15 ml per hour. The column was eluted with about 12 ml of the same Tris-HCl buffer containing 0.1 M KCl. The second step of the elution was carried out by applying about 12 ml of the same buffer

containing 0.25 M KCl. The elutions were monitored by measuring the A_{295} of the effluent. Both eluents were precipitated with ammonium sulfate (75% saturation) and dissolved in 750 µl of 1 mM DTT, 20 mM Tris-HCl pH 7.6. The fractions were dialyzed against the same buffer for 6 hours and then stored at -70°C. The 0.1 M KCl fraction contained the 58,000 dalton molecular weight protein and also contained some M2 subunit activity. The 0.25 M KCl fraction contained the 86,000 dalton protein and high M1 subunit activity.

Detection of M1 subunit by immunoprecipitation

Labelling of cellular proteins with ^{35}S-methionine: Culture plates (150 mm, Lux Scientific) that contained about 6 x 10^6 cells per plate were used. The medium was aspirated and the monolayer of cells was washed with PBS and then 4 ml of labelling medium was added. The labelling medium contained 80 µCi ^{35}S-methionine (800 Ci/mmol, NEN Research Product) per ml in Earle's modified methionine-free medium (Flow Laboratories) supplemented with 10% dialyzed FBS (Gibco). Cells were incubated for 16 hours at 37°C.

Preparation of ^{35}S-methionine labelled cell-free extract: The monolayer of labelled cells was rinsed with cold PBS and then detached from the plate with trypsin solution. The cells were washed twice with cold PBS and lysed by resuspending in solubilizing buffer (SB150) that contained 150 mM NaCl, 5 mM EDTA (Disodium Ethylenediamine Tetracetate, Sigma), 1% triton X-100, 0.5% sodium deoxycholate and 25 mM Tris-HCl pH 7.5. Solubilization was carried out at room temperature for 15 minutes. The extract was clarified by centrifugation in a microfuge at 12,000 x g for 10 minutes.

Preadsorption of ^{35}S-methionine labelled cellular extract: The cell free extract was preadsorbed with formalin fixed S. aureus cells (Pansorbin, Calbiochem. Ltd.). An aliquot of 20 ul of a 10% suspension was added to 200 1 of extract and incubated at room temperature with mixing for 15 minutes. The S. aureus cells were removed by centrifugation (12,000 x g, 2 minutes) and the preadsorbed extract was recovered. This procedure serves to reduce non-specific background signal by removing labelled cellular materials that can bind non-specifically to Pansorbin.

Preadsorption of S. aureus cells with cell free preparation of unlabelled mouse L cells: Cell free extract of non-labelled mouse L cells was prepared by resuspending 1 x 10^7 cells in 1 ml of SB150 buffer and incubated at room temperature for 15 minutes before removing cellular debris by centrifugation (15,000 x g, 20 minutes). An aliquot of a 10% suspension of S. aureus cells was mixed with 2x volume of the cell free extract and incubated at room temperature for 15 minutes. This treatment allows the unlabelled cell extract to interact with non-specific binding sites on the Pansorbin and thereby reduces the amount of non-specific binding between Pansorbin and radioactively labelled cell extract. The preadsorbed S. aureus cells were recovered by centrifugation and resuspended in SB250 buffer (SB150 with 250 mM NaCl) to make a 10% cell suspension. This cell suspension was used to precipitate the antigen-antibody complexes in the immunoprecipitation procedure.

Immunoprecipitation: Prior to immunoadsorption, a 5 1 sample from each labelled extract was treated with 10% trichloroacetic acid

(TCA) and the TCA-precipitable radioactivity was determined. The same number of TCA-precipitable counts were used in the subsequent immunoprecipitation. The immunoprecipitation was carried out according to the method of Firestone et al (1980). A volume of 200 ul of extract containing the desired amount of TCA-precipitable radioactivity was mixed with 5 ul of antiserum or control serum and 100 ul of SB250 solubilizing buffer containing 50 mg/ml of bovine serum albumin (BSA) (Sigma). After incubating at room temperature for 15 minutes, 15 µl of 10% suspension of preadsorbed S. aureus cells was added to the mixture and incubated for another 5 minutes. The entire reaction mixture was layered on 600 ul of 1 M sucrose solution in a microfuge tube. The mixture was centrifuged for 3 minutes at 12,000 x g. The top layer was aspirated and the side of the tube was washed with 2 M urea solution. The sucrose layer was aspirated and the S. aureus pellet was first washed with SB250 buffer and then with TE buffer (5 mM EDTA, 10 mM Tris-HCl pH 7.5). The precipitated proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

Polyacrylamide gel electrophoresis (SDS-PAGE): The immunoprecipitated proteins were analyzed by SDS-PAGE using the method of Laemmli (1970). The pellet of S. aureus cells containing the antigen was resuspended in 40 µl of sample loading buffer which contained 3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue and 625 mM Tris-HCl pH 6.8. The sample was denatured by heating in a boiling water bath for 3 minutes and insoluble materials were removed by centrifugation

(12000 x g, 2 minutes). The entire volume of the sample was electrophoresed on a 10% polyacrylamide gel at constant current (35 mA per gel) for about 4 hours. The polyacrylamide gel was composed of 10% (w/v) polyacrylamide (Bio Rad Laboratories), 1% SDS and 0.4 M Tris-HCl pH 8.8. The electrophoretic equipment used was a vertical slab gel electrophoretic apparatus (Protean, Bio Rad Laboratories). Gels were stained at room temperature for 4 hours with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma Chemicals), 10% acetic acid and 50% methanol. Destaining was carried out in 10% acetic acid and 50% methanol by simple diffusion. Gels were preserved by drying on a slab gel dryer (Model 1125B, Bio Rad Laboratories).

Autoradiography: The radioactively labelled proteins in the electrophoresed gel were detected using Kodak X-Omat PR film. The exposure was carried out at -70°C with Cronex 'lightning plus' intensifying screen (Du Pont). The film was developed in a Kodak PR X-Omat processor.

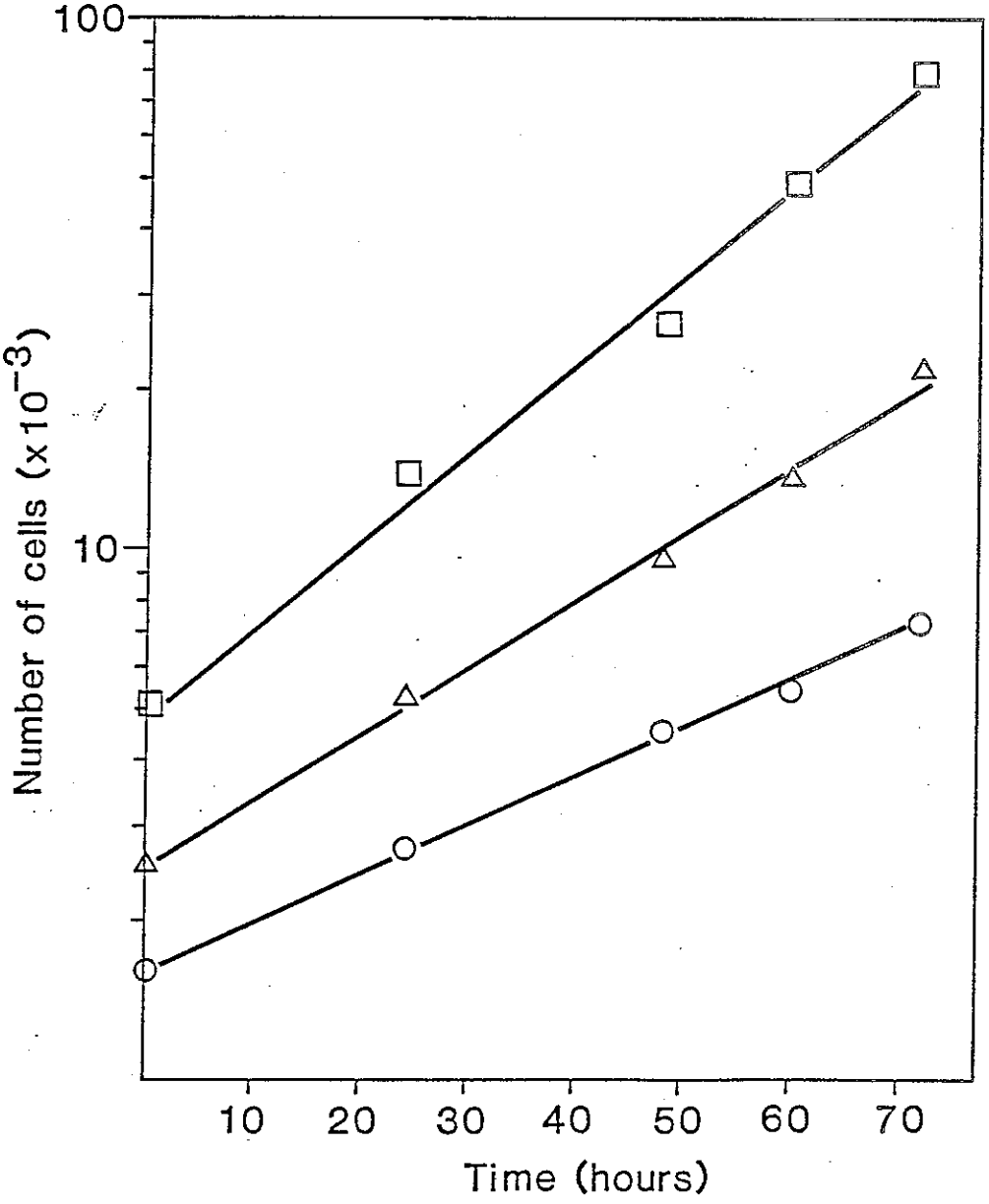
RESULTS

Growth Rates of Wild Type and LHF Cells Grown in the Absence and Presence of 5 mM Hydroxyurea.

A drug resistant mouse L cell line designated as LHF was selected by culturing cells in the presence of stepwise increasing concentrations of hydroxyurea (up to 5 mM drug) as described in Materials and Methods. The LHF stock culture was continuously grown in the presence of 5 mM hydroxyurea and was called LHF^+, whereas LHF cells that were grown in the absence of hydroxyurea for at least 40 doublings were called LHF^-. A study on the growth rates of wild type, LHF^- and LHF^+ cells was performed as described in Materials and Methods.

Figure 7 shows that the wild type (WT) cells exhibited the fastest growth rate; with a doubling time of about 18 hours. The LHF^- cell line, which had been growing in the absence of hydroxyurea for more than 5 passages, showed a doubling time of approximately 24 hours. The doubling time for LHF^+ cells (grown in the presence of 5 mM hydroxyurea) was found to be approximately 33 hours. It appeared that the presence of 5 mM hydroxyurea reduced the growth rate of LHF cells by 27 percent.

Figure 7. A comparison of the logarithmic growth rate of wild type mouse L cells (□), drug resistant LHF cells grown in the absence (△) and presence of 5 mM hydroxyurea (○). The cells were grown on 60 mm plastic tissue culture plates as described in Materials and Methods.

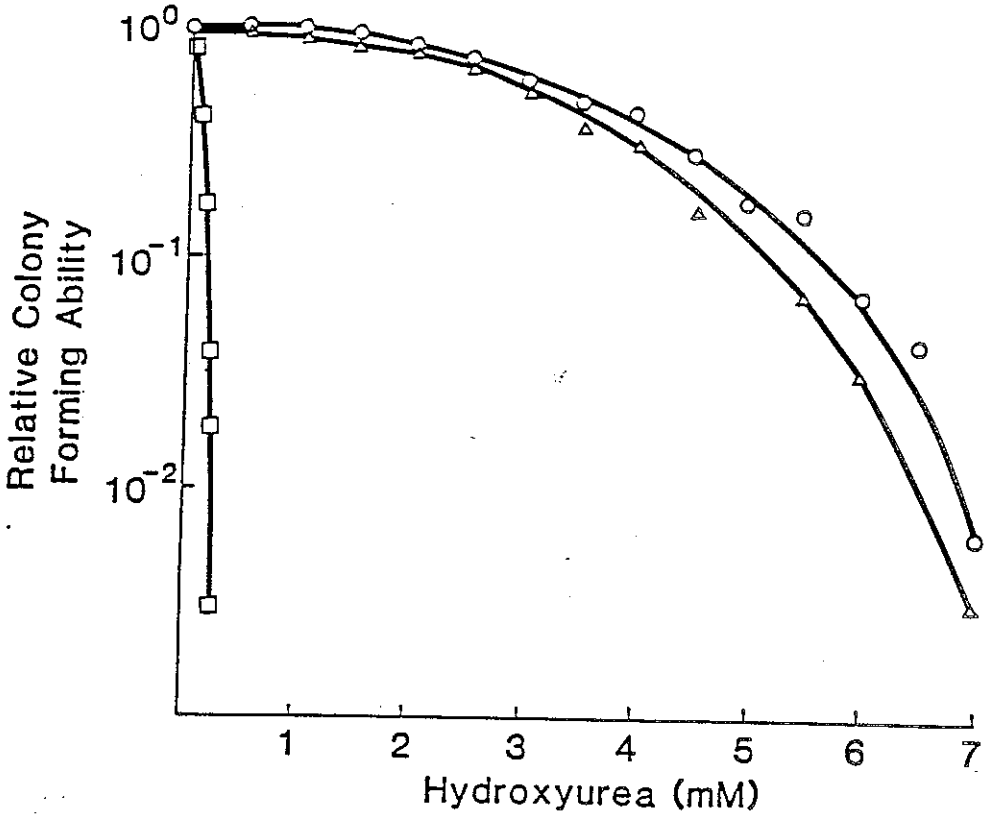

100
10
Number of cells (x10^{-3})
10
20
30
40
50
60
70
Time (hours)

Colony-forming Ability in the Presence of Hydroxyurea.

The sensitivity of WT mouse L cells and the drug resistant (LHF) cells to hydroxyurea was compared by examining their ability to form colonies in the presence of hydroxyurea. LHF cells that were grown in the absence of hydroxyurea for more than 40 doublings were tested for the stability of the resistant phenotype (LHF^-).

Figure 8 shows the relative colony-forming ability of WT cells and LHF cells in the presence of various concentrations of hydroxyurea. The result clearly indicated that LHF^+ and LHF^- cells were significantly more resistant to the cytotoxic effects of the drug than the WT cells. The D_{10} values (the concentration of drug at which the relative plating efficiency is 10 percent) for WT, LHF^- and LHF^+ cells were 0.17 mM, 5.2 mM and 5.5 mM respectively. By comparing their D_{10} values, the LHF^- and LHF^+ cells were approximately 30-fold more resistant to hydroxyurea than WT cells. It is important to note that LHF cells grown in the absence of hydroxyurea for more than 40 doublings retained a level of resistance to the drug almost identical to that of LHF^+ cells. This result indicates that the hydroxyurea resistant phenotype is stable even when the cells are grown in the absence of the drug for extended periods of time.

Figure 8. Relative colony forming abilities of wild type (□), drug resistant LHF cells that were grown in the absence of hydroxyurea (△), and LHF cells that were grown continuously in the presence of 5 mM hydroxyurea (○). The co-ordinate is in logarithmic scale.


Relative Colony Forming Ability
10^0
10^{-1}
10^{-2}
1
2
3
4
5
6
7
Hydroxyurea (mM)

Effects of Hydroxyurea on Ribonucleotide Reductase Activity.

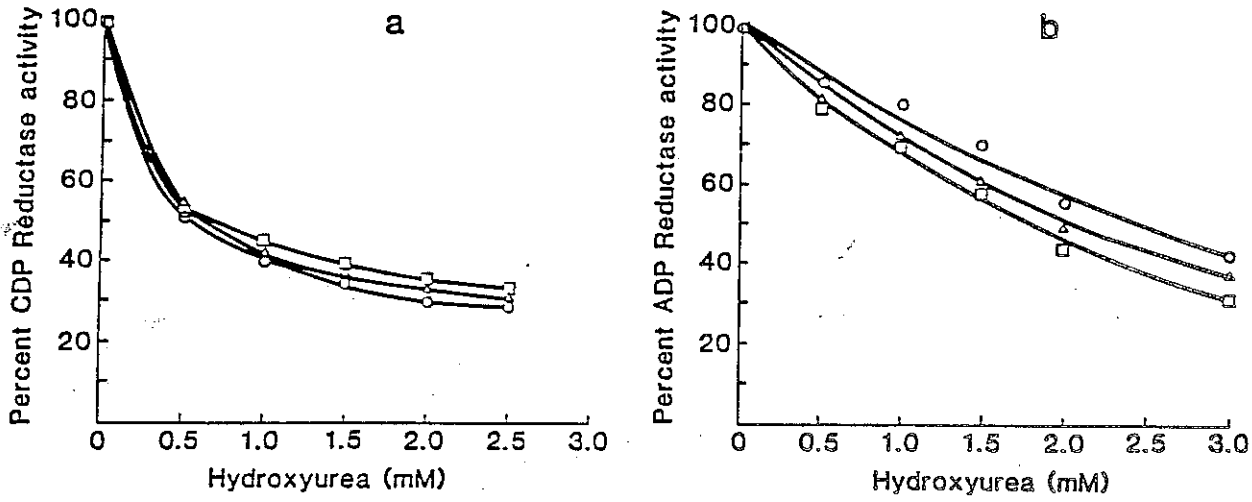
According to previous biochemical studies, alterations in ribonucleotide reductase activity in drug resistant cell lines can be classified into three general classes (Wright 1983; Wright et al, 1980; Wright et al, 1981). The first class of drug resistant cells contain an enzyme activity less sensitive to drug inhibition, a second class possesses elevated levels of reductase activity but with a WT sensitivity to drug. A third class of resistant cells contain both of the alterations described above.

It was of interest to characterize the LHF cell line and to determine whether the ribonucleotide reductase activity was less sensitive to hydroxyurea inhibition. The permeabilized cell assay was used to measure the rate of cytidine diphosphate (CDP) and adenosine diphosphate (ADP) reduction in WT, LHF^- and LHF^+ cells, in the presence of varying concentrations of hydroxyurea. The results shown in Figure 9 indicated that there was no significant difference between the cell lines in sensitivity of their intracellular CDP and ADP reductase activities to hydroxyurea. As shown in Figures 9a and 9b, the 50% inhibition of CDP and ADP reduction in WT and resistant cells occurred between 0.5 mM and 0.53 mM, and between 1.8 mM to 2.5 mM, respectively. This suggests that the reduction of CDP is more sensitive to hydroxyurea inhibition than ADP reduction. Since there was no apparent difference in hydroxyurea sensitivity between ribonucleotide reductase from WT and LHF cells, it seems unlikely that the drug resistant properties of the LHF cell line could be due to an alteration in the M2 subunit of the reductase.

Figure 9a and b.

a) Inhibitory effect of various concentrations of hydroxyurea on CDP reductase activity of permeabilized WT (□), LHF$^-$ (△) and LHF$^+$ (○) cells expressed as percent of activity in the absence of hydroxyurea.

b) Inhibitory effect of various concentrations of hydroxyurea on ADP reductase activity of permeabilized WT (□), LHF$^-$ (△) and LHF$^+$ (○) cells expressed as percent of activity in the absence of hydroxyurea.


a
Percent CDP Reductase activity
100
80
60
40
20
0
0.5
1.0
1.5
2.0
2.5
3.0
Hydroxyurea (mM)
b
Percent ADP Reductase activity
100
80
60
40
20
0
0.5
1.0
1.5
2.0
2.5
3.0
Hydroxyurea (mM)

Effects of Deoxyadenosine Triphosphate on Ribonucleotide Reductase Activity.

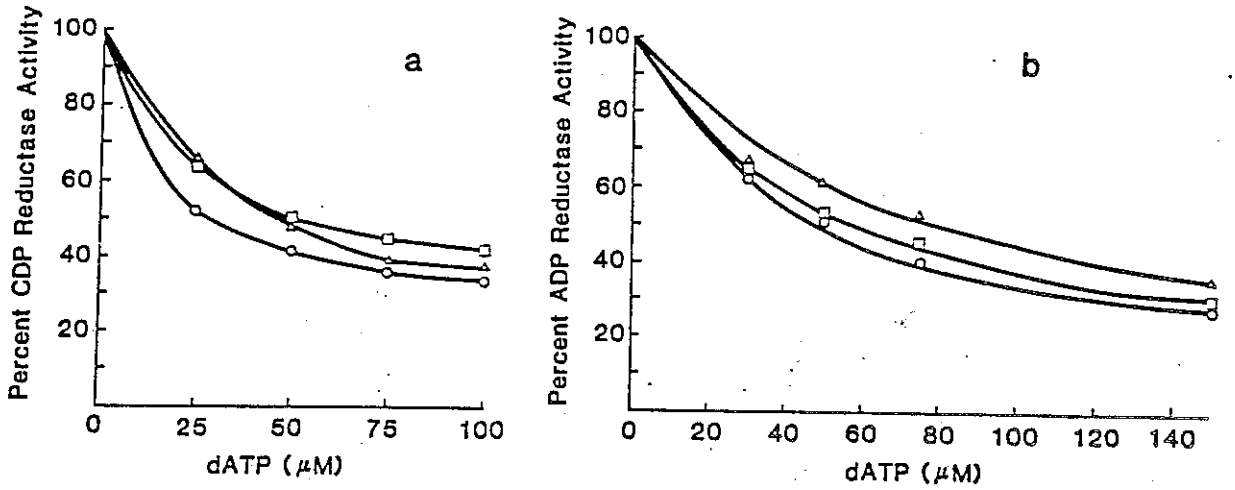
Both the substrate specificity and activity of ribonucleotide reductase are under strict allosteric regulation by a variety of effectors (Thelander and Reichard, 1979; Wright, 1983, 1987). Enzyme activity and regulation is mediated by the binding of specific nucleotides to three genetically defined sites on the M1 or effector-binding subunit (Eriksson et al, 1981a,b; Eriksson, 1982; Ullman, 1979, 1980, 1981). Deoxyadenosine triphosphate (dATP) is an overall negative effector which can bind to the activity site on the M1 subunit and consequently reduce the activity of the reductase. Ullman et al (1980) reported isolating a cell line that was partially desensitized to the negative effector, dATP. In theory, such cell lines could display increased levels of ribonucleotide reductase activity in vivo, since the enzyme would respond less stringently to intracellular dATP pool regulation. In order to determine if there was a significant modification in dATP inhibition of CDP and ADP reductase activities in LHF cells, assays were performed in the presence of various concentrations of dATP (Figure 10). The results of these experiments indicated that there was very little difference between wild type and drug resistant cells with regard to dATP sensitivity. Reduction of CDP by the three cell lines was inhibited to the 50% level between 27 and 52 μM dATP, and ADP reduction was reduced to 50% inhibition between 50 μM and 80 μM dATP. Ribonucleotide reductase activities of the WT, LHF^- and LHF^+ cells displayed similar sensitivity to dATP inhibition suggesting that no

obvious alteration to dATP response had occurred, and enzyme sensitivity did not appear to be altered in LHF cells (LHF^{+}) previously cultured in the presence of hydroxyurea.

Figure 10a and b.

a) Inhibitory effect of various concentrations of dATP on CDP reductase activity of permeabilized WT (□), LHF$^-$ (△) and LHF$^+$ (○) cells expressed as percent of activity in the absence of dATP.

b) Inhibitory effect of various concentrations of dATP on ADP reductase activity of permeabilized WT (□), LHF$^-$ (△) and LHF$^+$ (○) cells expressed as percent of activity in the absence of dATP.


Percent CDP Reductase Activity
100
80
60
40
20
0
25
50
75
100
dATP (μM)
a
Percent ADP Reductase Activity
100
80
60
40
20
0
20
40
60
80
100
120
140
dATP (μM)
b

Intracellular Ribonucleotide Reductase Activity Levels in Wild-type and Hydroxyurea Resistant Cells.

During the drug sensitivity studies of ribonucleotide reductase activity of wild-type and LHF cell lines, it was observed that the LHF cells consistently demonstrated a much higher enzyme activity. This observation supported the possibility that the hydroxyurea resistant cells overproduced ribonucleotide reductase activity to neutralize the effect of the drug. Therefore, a more detailed study of enzyme activity was carried out by measuring the formation of dCDP and dADP as a function of increasing substrate concentration in permeabilized cells.

Figure 11a and b clearly indicate that the CDP and ADP reductase activities were markedly elevated in the LHF cells. From Figure 11a, the amount of dCDP formed in the presence of 2 mM CDP (a saturating substrate concentration) by WT, LHF^- and LHF^+ cells was 1.0, 5.5 and 18.5 nmol dCDP/3 x 10^6 cells/h respectively. LHF^- and LHF^+ cells demonstrated approximately a 5 fold and 18 fold increase in CDP reductase activity respectively. Similar differences in ADP reductase activity were also observed (Figure 11b). The amount of dADP formed in the presence of 2 mM ADP by WT, LHF^- and LHF^+ cells was 1.1, 3.3 and 12.0 nmol dADP/3 x 10^6 cells/h respectively. Therefore, LHF^- and LHF^+ cells showed increases of about 3 fold and 12 fold in ADP reductase activity respectively.

When the results shown in Figures 11a and b were analyzed further by the Lineweaver-Burk plot (Figures 12a and b) the differences in enzyme levels between the various cell cultures were again obvious,

and no significant differences in km values for CDP or ADP were observed (Table 1). The similarity in substrate km values supports the results of enzyme inhibition studies (Figures 9 and 10), which suggested that ribonucleotide reductase in LHF cells was not structurally altered.

Figure 11a and b.

a) The velocity of CDP reduction in the presence of varying concentrations of CDP in permeabilized wild type mouse L cells (□), LHF^- cells (△) and LHF^+ cells (○).

b) The velocity of ADP reduction in the presence of varying concentrations of ADP in permeabilized wild type mouse L cells (□), LHF^- cells (△) and LHF^+ cells (○).

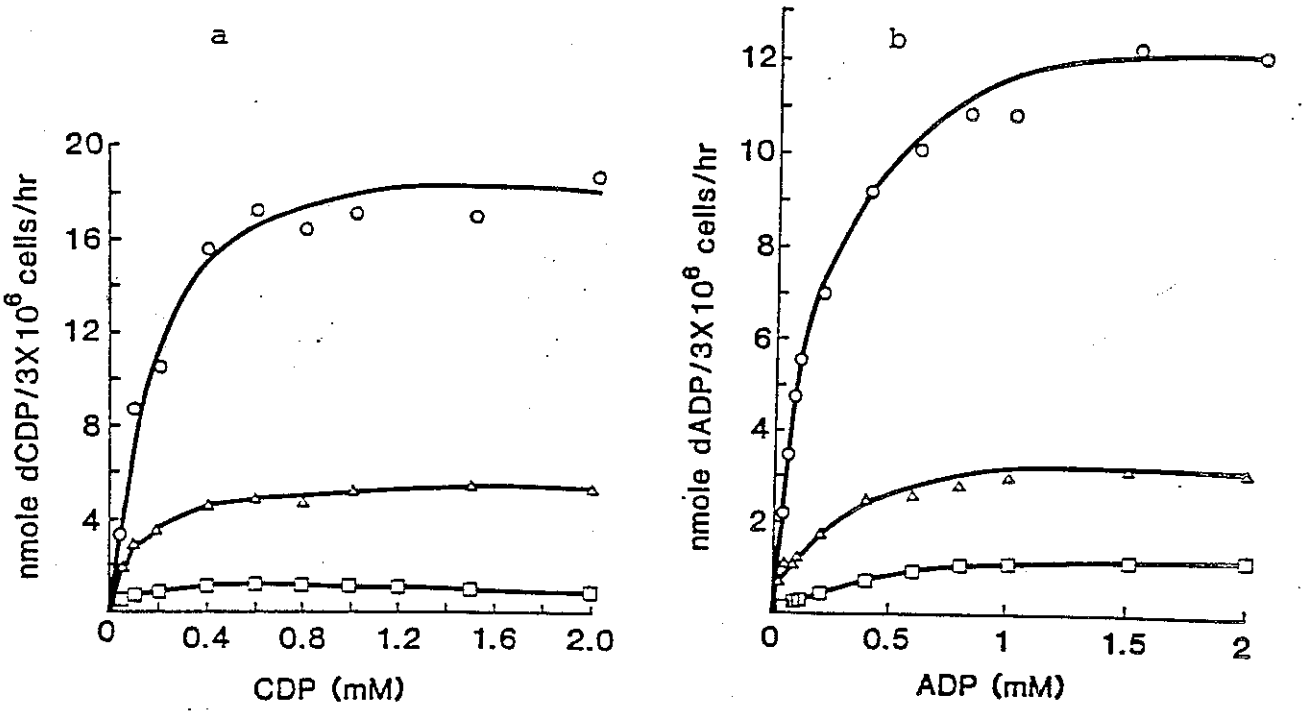
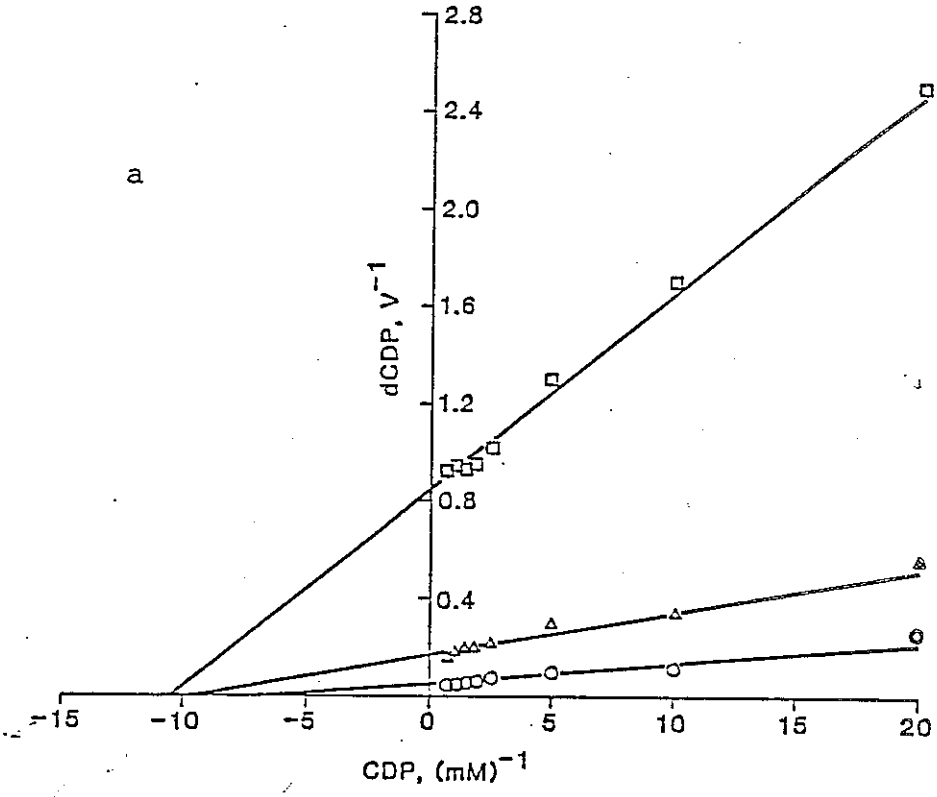
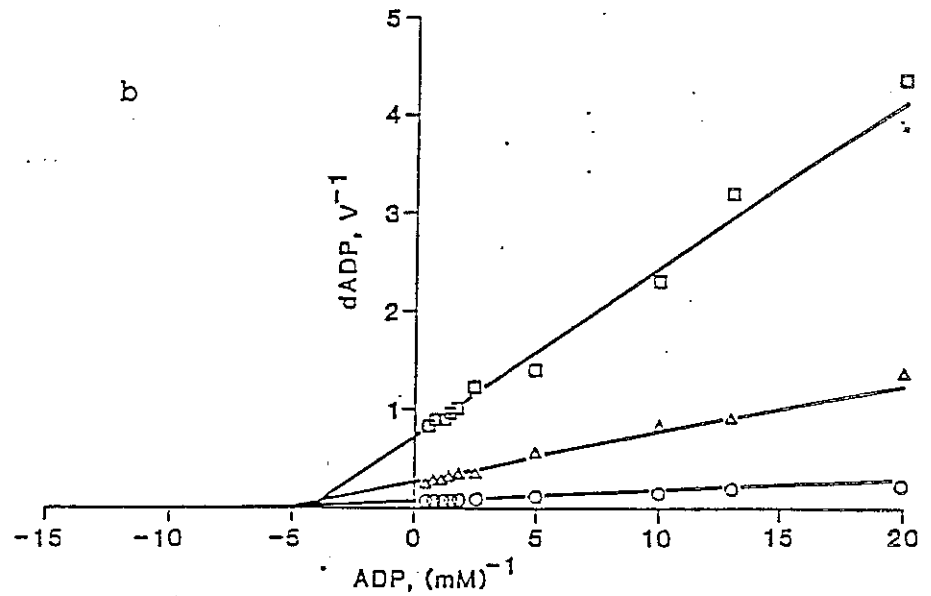

a
nmole dCDP/3X10^6 cells/hr
20
16
12
8
4
0
0.4
0.8
1.2
1.6
2.0
CDP (mM)
b
nmole dADP/3X10^6 cells/hr
12
10
8
6
4
2
0
0.5
1
1.5
2
ADP (mM)

Figure 12a and b.

a) Lineweaver-Burk plot of velocity against increasing concentration of CDP in wild type mouse L cells (□), LHF^- cells (△) and LHF^+ cells (○).

b) Lineweaver-Burk plot of velocity against increasing concentration of ADP in wild type mouse L cells (□), LHF^- cells (△) and LHF^+ cells (○).


a
2.8
2.4
2.0
1.6
1.2
0.8
0.4
dCDP, V⁻¹
-15
-10
-5
0
5
10
15
20
CDP, (mM)⁻¹


b
5
4
3
2
1
dADP, V⁻¹
-15
-10
-5
0
5
10
15
20
ADP, (mM)⁻¹

Variations in ribonucleotide reductase activity in LHF cells: time dependence.

The following series of experiments were aimed at studying the changes in ribonucleotide reductase activity in LHF cells as they were transferred from hydroxyurea-supplemented medium to hydroxyurea-free medium and vice versa. Previous studies (Figure 11) have shown that when LHF cells were cultured in the absence of hydroxyurea for an extended period of time, the enzyme activity decreased by 75%. However, this decrease in enzyme activity was not accompanied by a similar decrease in hydroxyurea resistance (Figure 8).

The rate of decrease in CDP reductase activity after hydroxyurea was removed from the culture medium was examined. At several time points after growing cells in the absence of drug, CDP reductase activity was assayed in a cell-free preparation. The results obtained with cell-free preparations were consistent with those obtained with permeabilized cells. In these experiments, we consistently observed about a 20-fold increase in CDP reductase activity with LHF^+ cells, when compared to the WT population. For example, in three determinations, we observed 1.8 ± 0.4, 8.0 ± 1.7 and 37.3 ± 6.2 nmol dCDP/mg/h with enzyme preparations from WT, LHF^- and LHF^+ cells respectively. These observations closely resembled the results of CDP reductase assays in permeabilized cells (Table 1).

Table 2 shows the CDP reductase activity of LHF cells when cultured in the absence of hydroxyurea up to a period of 12 months. After 24 hours, the enzyme activity was found to be 82% of the initial level (18% reduction). At the end of 54 hours, the enzyme activity

was at 33% of the original level. This represented a more substantial reduction of 49% in enzyme activity between 24 and 54 hours. The level of enzyme activity remained at about 27% even after culturing in the absence of drug for 12 months.

In a complementary experiment, we examined the kinetics of enzyme induction by hydroxyurea (Figure 13). LHF cells continuously cultured in hydroxyurea-containing medium were grown in the absence of drug for 2 days and then returned to hydroxyurea-containing medium. At several time points after the replacment of drug, CDP reductase activity of these cells was measured by the permeabilized cell assay system. Figure 13 showed the level of enzyme activity relative to cells that were continuously grown in the presence of hydroxyurea. After culturing in the absence of drug for 2 days, the CDP reductase activity was at about 30% of the activity observed when cells were cultured continuously in the presence of hydroxyurea. Upon returning to hydroxyurea supplemented medium, the level of CDP reductase activity in the LHF cells recovered rapidly, rising to approximately 75% of enzyme activity in LHF^+ cells after 18 hours. This result suggests that CDP reductase activity in these cells is inducible in the presence of hydroxyurea.

Table 1. Summary of the km and V_{max} values of CDP and ADP reductase activity in WT, LHF^- and LHF^+ cells.

Cell lines	Km for substrates (mM)		V_{max} nmoles dNTP/$3x10^6$ cells/hr	
	CDP	ADP	CDP reduction	ADP reduction
Wild type cells	0.096±.03	0.23±.05	1.12±.11	1.3±.15
LHF^- cells	0.11 ±.01	0.18±.01	5.7 ±.12	3.5±.06
LHF^+ cells	0.15 ±.03	0.15±.01	20.0 ±.86	13.0±.25

The km and V_{max} values were obtained with permeabilized cells described in Materials and Methods and the data was analyzed using a computerized statistical analysis devised by Cleland (1967).
The data shown were calculated from three independent determinations.

Table 2. LHF CDP reductase activities[a] at various times after removal from growth medium containing 5 mM hydroxyurea.

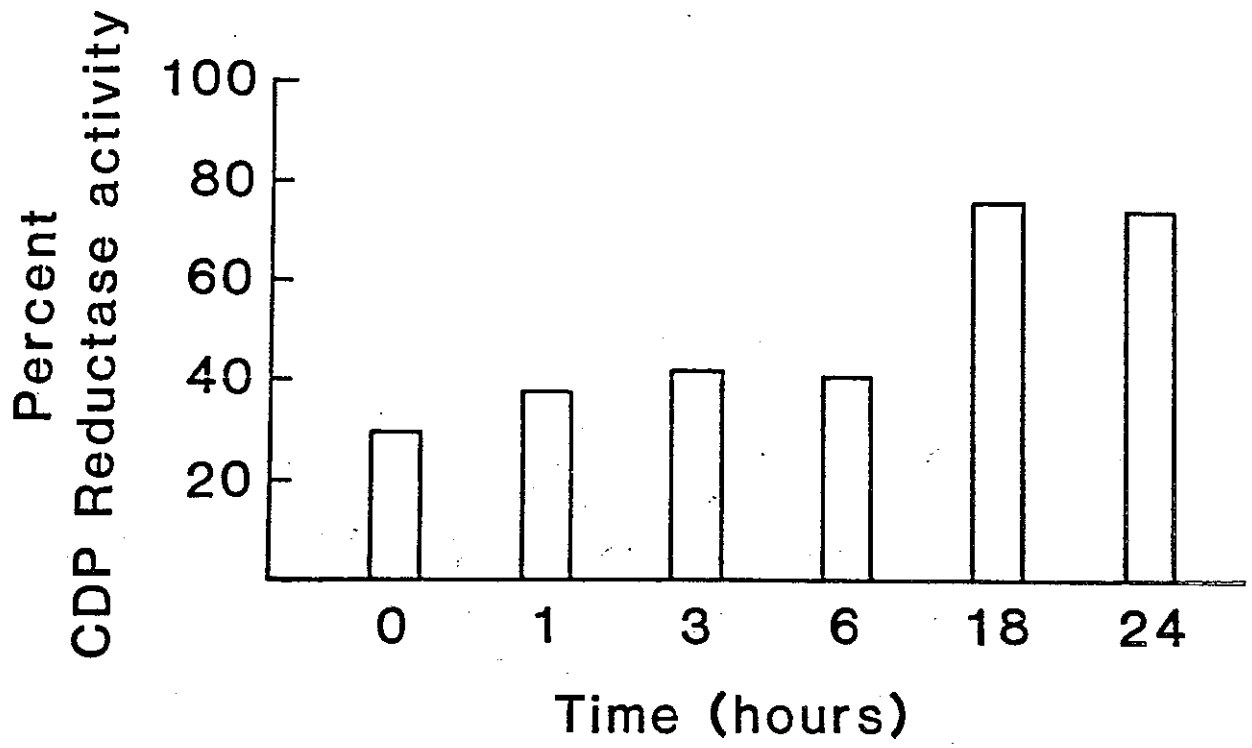
Time[b]	nmole/hr/mg ± S.E.[c] Protein	% Activity Remaining
5 hr	30.5 ± 4.3	95
19 hr	27.1 ± 3.5	85
24 hr	26.2 ± 3.9	82
54 hr	10.6 ± 2.8	33
6 months	8.7 ± 2.3	28
1 year	8.4 ± 2.4	27

[a]activity determined in cell free preparations; LHF cells grown continuously in the presence of 5 mM drug reduced 32 nmoles CPB/hr/mg protein (100%).

[b]time after medium containing hydroxyurea was replaced with drug-free medium.

[c]data obtained from at least 3 determinations.

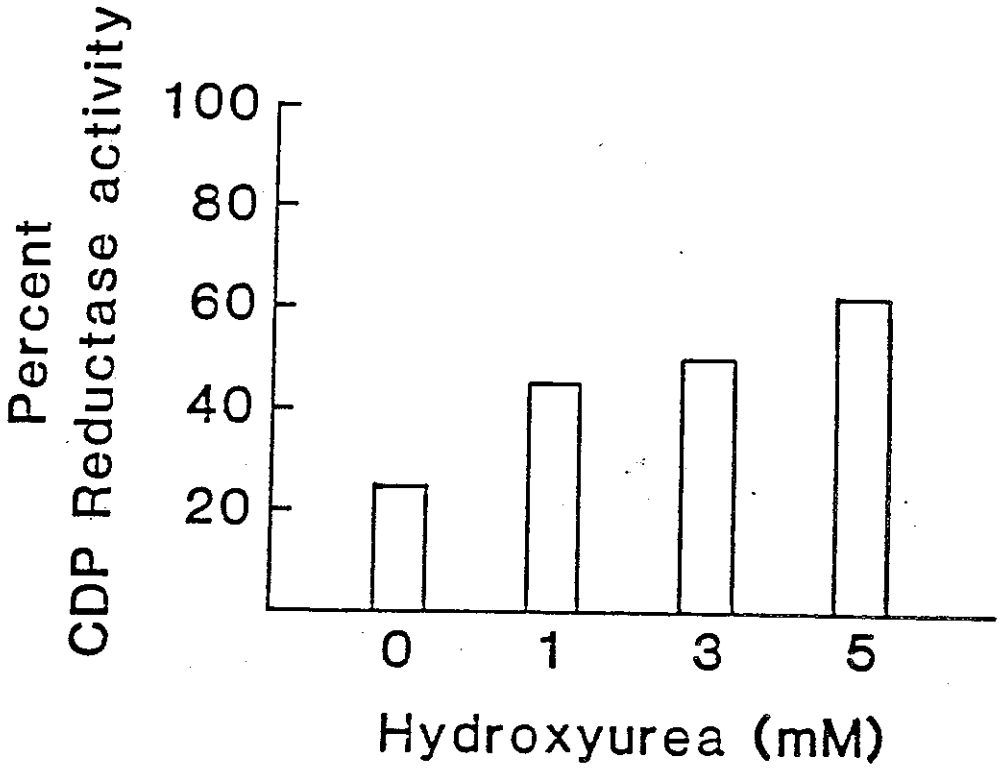
Figure 13. CDP reductase activity levels in permeabilized LHF^+ cells that were grown in the absence of hydroxyurea for 2 days and then were returned to growth medium containing 5 mM hydroxyurea for various lengths of time prior to enzyme assay.
100% = 5.2 nmole dCDP/10^6 cell/hr corresponding to the enzyme activity level in LHF^+ cells continuously grown in the presence of 5 mM hydroxyurea.


Percent
CDP Reductase activity
100
80
60
40
20
0
1
3
6
18
24
Time (hours)

Variations in Ribonucleotide Reductase Activity in LHF cells: Drug Dose Dependence.

The effect of hydroxyurea concentration on the induction of CDP reductase activity was studied by culturing LHF^+ cells in the absence of drug for 48 h, and then the drug-free medium was replaced with medium containing various concentrations of hydroxyurea for 16 h prior to enzyme assay. CDP reductase activity was measured in permeabilized cells and expressed as percent of enzyme activity in LHF cells continuously cultured in the presence of drug (Figure 14). The results indicated that the actual increase in CDP reductase activity depended upon the concentration of hydroxyurea. For example, the percent of maximum elevation of enzyme activity for a 16 hour incubation in the presence of 1 mM hydroxyurea was 45%, and in the presence of 5 mM hydroxyurea it was 65%.

Figure 14. CDP reductase activity levels in permeabilized LHF^+ cells that were grown in the absence of hydroxyurea for 2 days then were returned to growth medium containing various concentrations of hydroxyurea for 16 hours prior to enzyme assay.
100% = 4.6 nmole dCDP/10^6 cells/hr corresponding to the enzyme activity level in LHF^+ cells continuously grown in the presence of 5 mM hydroxyurea.


Percent
CDP Reductase activity
100
80
60
40
20
0
1
3
5
Hydroxyurea (mM)

Subunits of Ribonucleotide Reductase in Wild-type and LHF Cells.

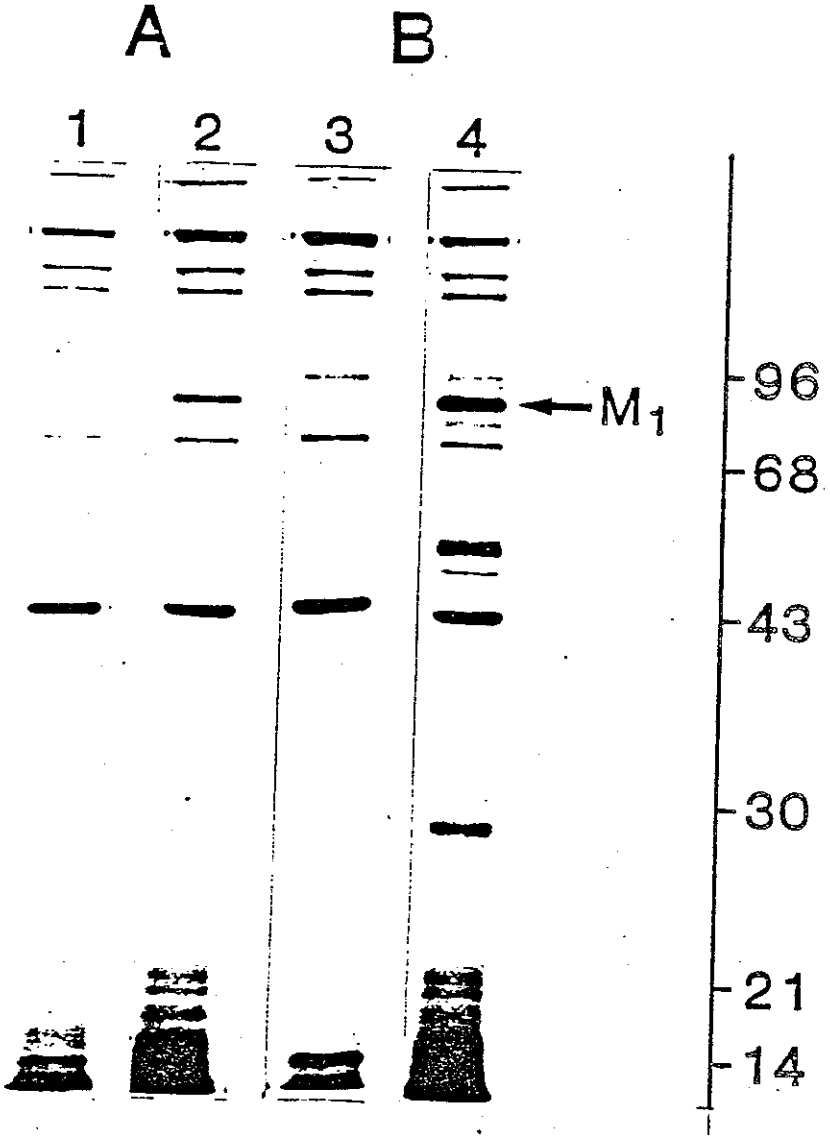
LHF cells exhibited a significant increase in ribonucleotide reductase activity when compared to WT cells. It was of interest to investigate the mechanism behind such elevation in enzyme activity.

Mammalian ribonucleotide reductase consists of two dissimilar subunits, often called M1 and M2, both of which are required for enzyme activity (Thelander and Reichard, 1979; Cory et al, 1983; Wright, 1983). The two subunits combine to form an active complex, therefore, the enzyme activity is limited by the less abundant of the two subunits.

An increase in enzyme activity could be due to one of the following factors: (1) an increase in M1 subunit, (2) an increase in M2 subunit, (3) an increase in both M1 and M2 subunits and (4) activation of pre-existing enzyme. There have been several reports describing hydroxyurea resistant cell lines showing elevated levels of ribonucleotide reductase activity (eg. Lewis and Wright, 1979; Wright et al 1980, 1981; Akerblom et al 1981; Wright 1983; Wright et al, 1987a; Lewis et al 1983; McClarty et al, 1986a). It has generally been assumed that increased enzyme activity is primarily due to increased levels of the M2 subunit, which contains a tyrosine free radical, and is the actual site of action for hydroxyurea (Thelander et al, 1985). To test the hypothesis that elevation of enzyme activity in LHF cells is a result of an overproduction of one or both of the subunits, we attempted to compare the levels of each of the reductase subunits in the wild type and LHF cells.

The amount of M1 subunit present in cell extracts was measured by

Figure 15. Immunoprecipitation of ^{35}S-methionine labelled cellular proteins from wild type mouse L cells (A) and LHF$^-$ cells (B) by incubation with preimmune serum (lanes 1 and 3) or anti-M1 antiserum (lanes 2 and 4) followed by adsorption to formalin fixed S. aureus cells and analysis by SDS 10% polyacrylamide gel electrophoresis (MW markers x 10^3). Besides M1, two other bands of lower molecular weight were increased in LHF cells and were probably degradation products of M1.

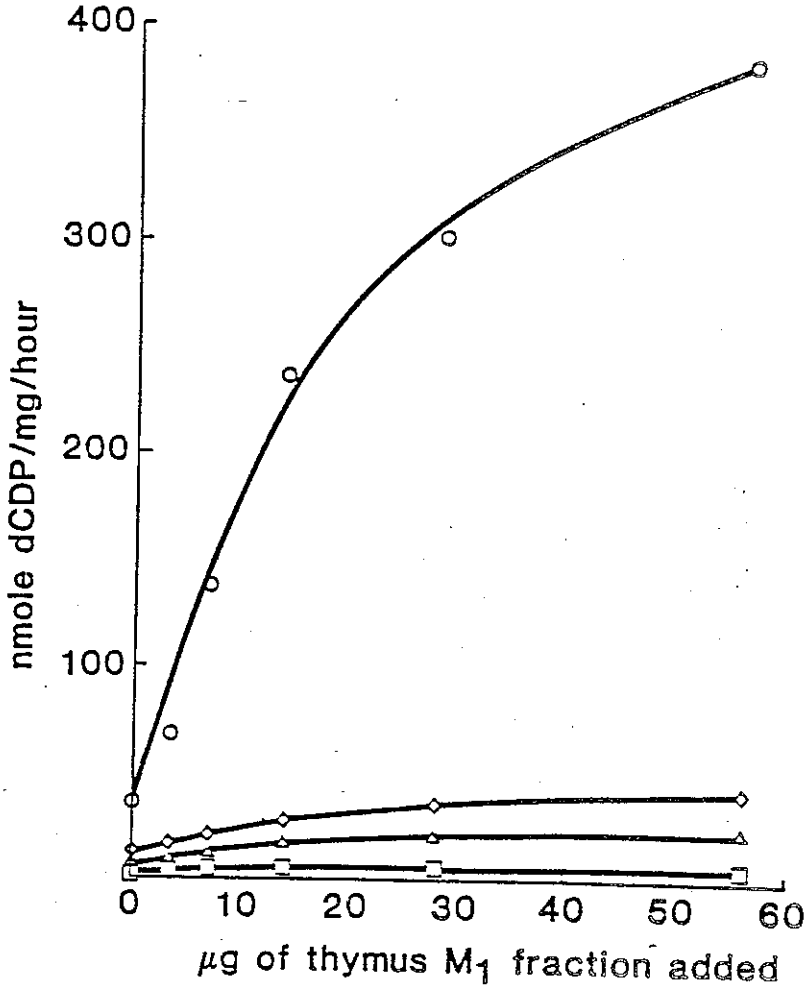

A
B
1
2
3
4
M1
96
68
43
30
21
14

an immunoprecipitation technique described in Materials and Methods. Cellular proteins were radioactively labelled by culturing cells in a ^{35}S-methionine containing medium for 16 hours. A relatively long labelling time was used, so that the result would reflect the intracellular steady state level of the M1 subunit. The M1-specific rabbit antiserum was kindly provided by B.B. Levinson and D.W. Martin, Jr. of Genentech, Inc. The results obtained in this experiment (Figure 15) are not strictly quantitative because some variation in growth rates (Figure 7) and in protein synthesis rates was observed between WT, LHF$^-$ and LHF$^+$ cells. However, immunoprecipitation experiments performed with WT and LHF$^-$ cell lines clearly showed that there was an increase in the concentration of the M1 subunits in LHF$^-$ cells (Figure 15).

A direct quantitation of the M2 subunit was not carried out because M2-specific antibody was not available at the time of this study. However, it has been shown that mouse M2 subunit is complemented by M1 subunit purified from thymus tissue (Akerblom et al, 1981), making it possible to compare M2 activities in various cell lines by titration experiments (Cory and Fleischer, 1982; McClarty et al, 1986a). The M1 subunit was purified from lamb thymus as described in Materials and Methods. The titration experiment was carried out by adding increasing amounts of purified M1 subunit to a cell-free preparation until the saturating level was reached. The ribonucleotide reductase activity assayed in the presence of excess M1 subunit provided an indication of the amount of M2 subunit activity contained in the cell-free preparation.

The results (Figure 16) showed that at about saturating levels of M1 subunit, extracts of LHF^- cells exhibited about 10-fold more M2 activity, and LHF^+ extracts exhibited about 100-fold more M2 activity than extracts of WT mouse cells. Also of interest is the M2 titration experiment carried out with LHF^+ cells that were removed from drug for 2 days prior to assay. These cells showed a dramatic 90% reduction in M2 activity when compared to control LHF^+ cells, at M1 saturation levels. These cells showed only about 1.7 fold higher M2 activity than LHF^- cells which had been cultured in the absence of drug for more than 2 weeks. This significant decrease in M2 activity is consistent with the dramatic reduction in holoenzyme activity (Table 1) after LHF^+ cells were cultured in the absence of hydroxyurea for more than 24 hours.

Figure 16. Determination of M2 activity in cellular extracts from wild type mouse L cells (□), LHF^- cells (△), LHF^+ cells (○), and LHF^+ cells grown in the absence of hydroxyurea for 2 days (◇) prior to enzyme assay. Ribonucleotide reductase activity was measured in assay mixtures containing a constant amount of cellular extract and increasing amounts of purified M1 protein from thymus.


400
300
200
100
nmole dCDP/mg/hour
0
10
20
30
40
50
60
μg of thymus M1 fraction added

Effects of Antimetabolites on Ribonucleotide Reductase Activity.

Experimental results presented earlier have shown that ribonucleotide reductase activity in LHF cells could be elevated in response to the presence of hydroxyurea. Antimetabolites were used to investigate the mechanism by which enzyme activity was elevated. Actinomycin D and cycloheximide are antimetabolites that inhibit RNA synthesis and protein synthesis, respectively. These two reagents were used to test whether or not the elevation of enzyme activity required de novo synthesis of RNA or protein.

Table 3 shows the CDP reductase activity of LHF cells when grown in the absence or presence of hydroxyurea and antimetabolites. LHF cells that were grown in the presence of 5 mM hydroxyurea and removed from drug for 2 days showed enzyme activity of 12.6 nmoles dCDP/hr/mg protein. When these cells were re-exposed to 5 mM hydroxyurea for 6 hours, the level of activity increased to 19.0 nmoles dCDP/hr/mg protein or 151% of the uninduced control level. When these cells were incubated for 6 hr in the presence of only cycloheximide or actinomycin D (in the absence of hydroxyurea) an approximately 20% decrease in CDP reductase activity was observed. However, when the cells were simultaneously exposed to cycloheximide and hydroxyurea the increase in enzyme activity was prevented and the level of activity was similar to that of the uninduced control treated with cycloheximide alone. These results suggest that hydroxyurea induced elevation of CDP reductase activity is dependent on de novo protein synthesis. Furthermore, these results do not support the possibilities that the increase in reductase activity observed in LHF

cells grown in the presence of hydroxyurea were due to enzyme stabilization or activation by hydroxyurea.

The inhibitor of RNA synthesis, actinomycin D, also inhibited the hydroxyurea induced increase in reductase activity, showing only 65% of activity observed in cells treated with hydroxyurea alone. The results suggested that continuous de novo transcriptional activity was required for the increase in enzyme activity in response to the presence of hydroxyurea.

Table 3. Effects of cycloheximide and actinomycin D on enzyme activity.

Additions to Culture Medium	Enzyme Activity (nmoles dCDP/hr/mg protein) ± S.E.[a]
no additions	12.6 ± 1.1
+5 mM hydroxyurea	19.0 ± 1.5
+ cycloheximide	9.9 ± 1.5
+ cycloheximide and 5 mM hydroxyurea	9.3 ± 1.1
+ actinomycin D	10.2 ± 0.8
+ actinomycin D and 5 mM hydroxyurea	12.3 ± 1.3

[a]data obtained from at least 2 determinations.

Inhibition of Ribonucleotide Reductase Activity by Gossypol.

Gossypol is a yellow, polyhydroxylated binaphthalene compound isolated from the seed and bark of the cotton plant (Figure 17). It was isolated as long ago as 1886 but renewed interest in this compound was generated by the discovery that gossypol has antifertility effects in human males. The results of two separate clinical trials conducted in China have shown that gossypol was over 99% effective in inducing azoospermia and the effect was reversible in 90% of the subjects (Lau, et al 1981). Gossypol is now being used as a male contraceptive drug in some countries. Recognizing the potential importance of gossypol, many investigators examined various aspects of the biological effects of gossypol. It has been shown to interfere with a variety of biological functions. It was found to inhibit the (Ca^{2+} + Mg^{2+})-ATPase in sperm (Kalla and Vasudev, 1981) and the L-lactate dehydrogenase isoenzyme X in the testis (Morris et al, 1986). These two biological effects are probably related to the antifertility property of gossypol. It inhibits adenylate cyclase activity (Oligiati et al, 1984) and can also act as an uncoupler of mitochondrial oxidative phosphorylation (Abdou-Dania and Dieckert, 1974). It has been demonstrated that gossypol inhibited DNA synthesis in cultured mammalian cells even at relatively low concentrations (10 μg/ml) but RNA and protein synthesis were unaffected (Wang and Rao, 1984). Recently, Rosenberg et al (1986) demonstrated that DNA polymerase α of HeLa cells was inhibited by gossypol. Furthermore, antitumor activities of gossypol were demonstrated in certain experimental murine tumors (Tso, 1984; Rao, et al 1985). Gossypol was

Figure 17. Chemical structure of gossypol.

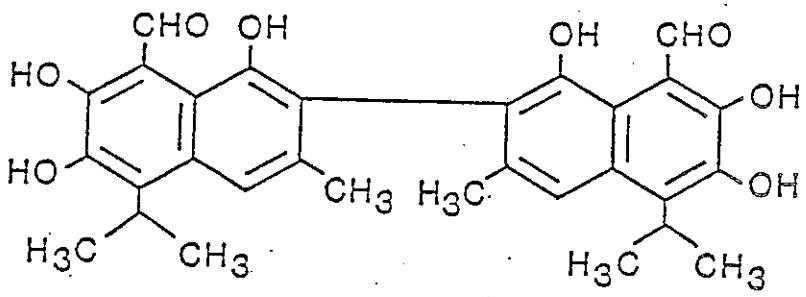
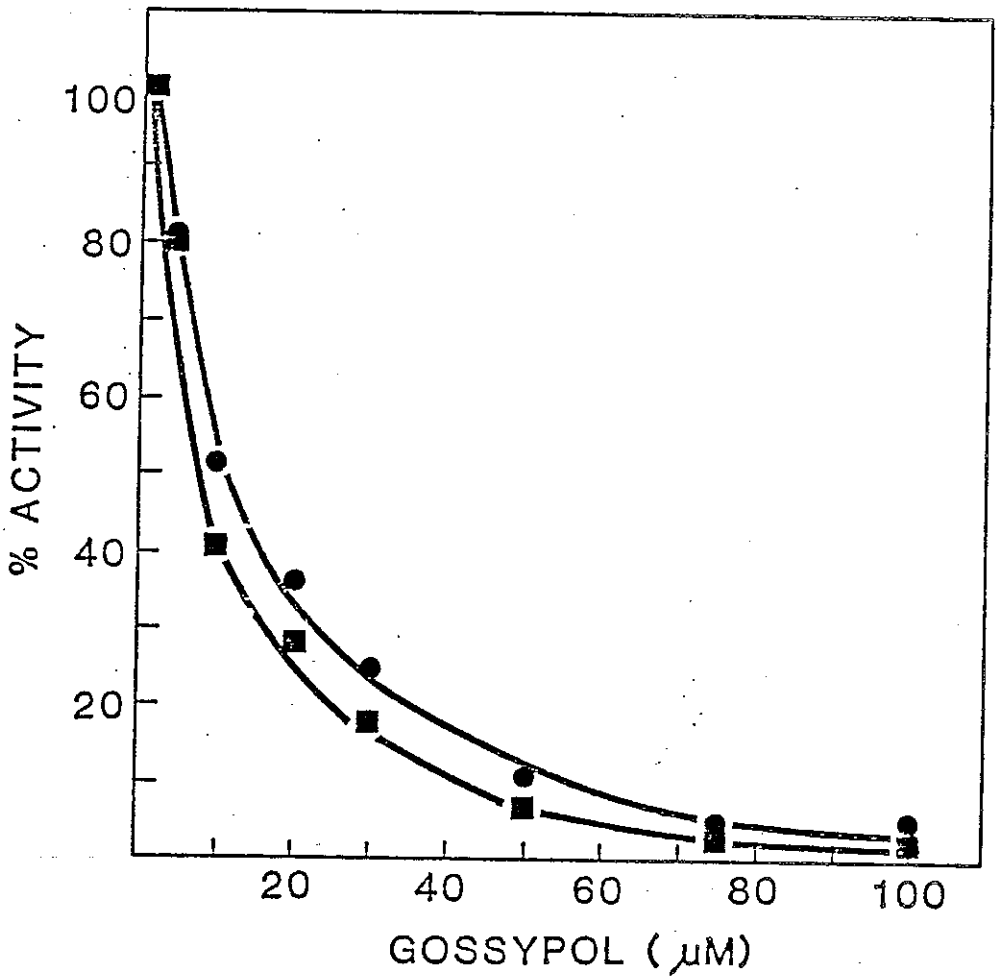

CHO
OH
HO
HO
CH3
H3C
CH3
OH
CHO
OH
OH
H3C
H3C
CH3

Figure 18. Ribonucleotide reductase activity measured in mouse L cell extracts in the presence of various concentrations of gossypol; CDP reduction (■) and ADP reduction (●). Enzyme activity is expressed as percent of activity in the absence of gossypol.

Velocity = nmole dNTP/mg/hr.


100
80
60
40
20
% ACTIVITY
20
40
60
80
100
GOSSYPOL (μM)

shown to be non-mutagenic according to the Ames test (Colman et al, 1979) and the sperm-head abnormality assay in mice (Majumdar et al, 1982). Chromosomal abberrations were not observed when cultured mammalian cells were treated with gossypol; however, a dose-dependent increase in DNA strand breaks and a slight increase in sister chromatid exchanges have been observed (Tsui et al, 1983). The chemical structure and other known biological activities of gossypol suggested that it could interfere with ribonucleotide reductase activity. This hypothesis was tested by determining ribonucleotide reductase activity in the presence of various concentrations of gossypol. Cell-free preparations of the LHF cell line provided an excellent source of ribonucleotide reductase activity for this study. The results of these experiments (Figure 18) indicated that gossypol is a potent inhibitor of ribonucleotide reductase activity. The rates of CDP and ADP reduction were reduced by 50% at 8 μM and 10 μM of gossypol respectively, indicating that both CDP and ADP reduction were similarily sensitive to gossypol inhibition.

A more detailed kinetic study of the effect of gossypol on the reduction of CDP and ADP was carried out (Figures 19 and 20). Lineweaver-Burk double reciprocal plot analysis showed that gossypol had very little effect on the apparent V_{max} values but dramatically altered the km values of the two substrates, suggesting that the drug inhibits the reduction of purines and pyrimidines in a competitive manner. Further analysis of the slopes of the double reciprocal plots with respect to gossypol concentration indicated that the K_i values for CDP and ADP reductions were 1.7 μM and 5.5 μM respectively (Inset

of Figures 19 and 20). The K_i value of gossypol is 1-2 orders of magnitude lower than hydroxyurea (Kuzik & Wright 1980; Hards & Wright, 1981) and is similar to that of the very potent thiosemicarbazones (Cory et al 1981; Thelander and Graslund, 1983).

Figure 19. Double reciprocal plots of velocity against CDP concentrations in mouse L cell extracts at several fixed concentrations of gossypol.
Activity in the absence (■) or presence of 10 μM (▲) and 20 μM (●) gossypol.
Inset: replot of slopes against drug concentrations.
Velocity = nmole dCDP/mg/hr

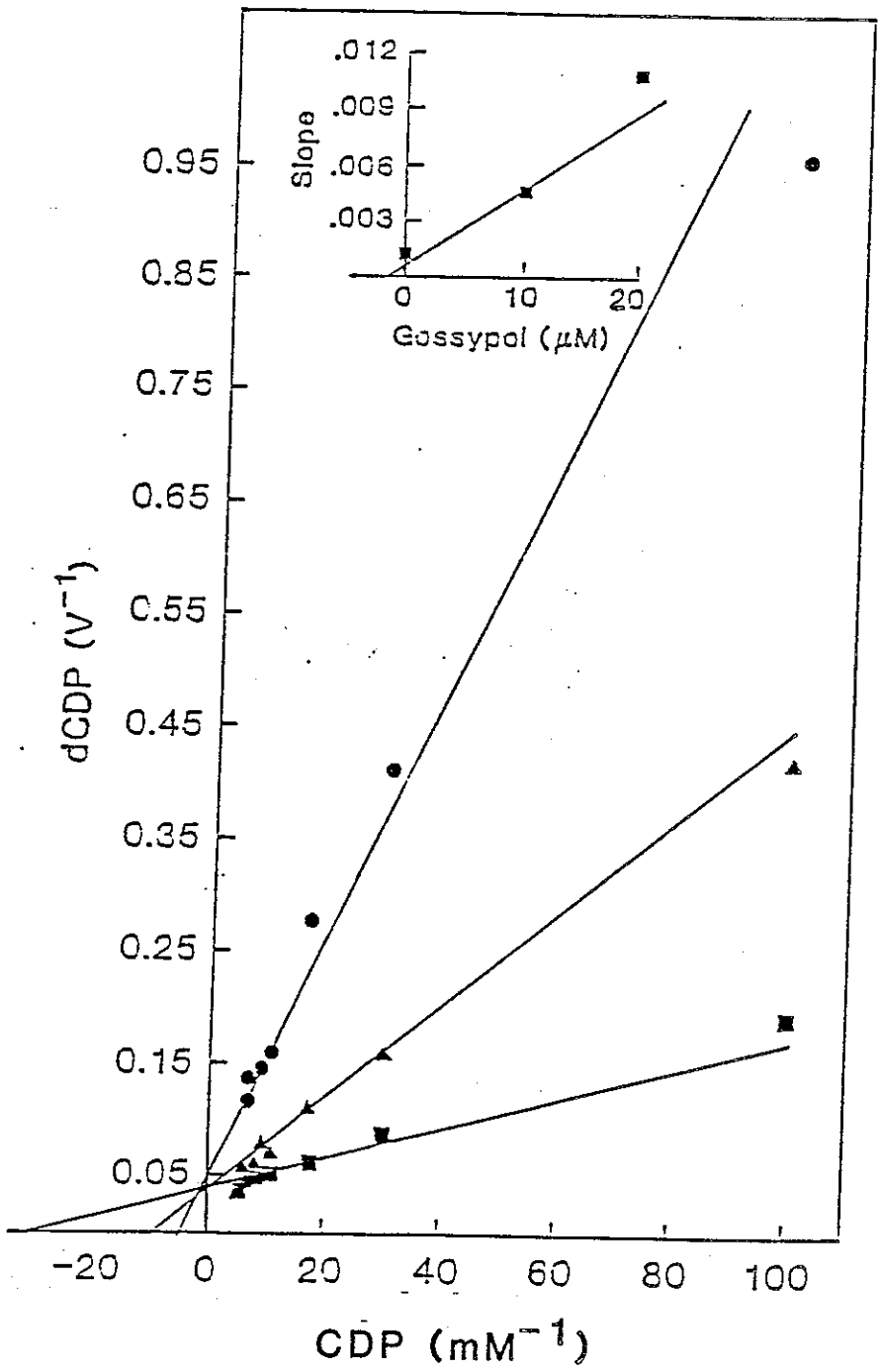
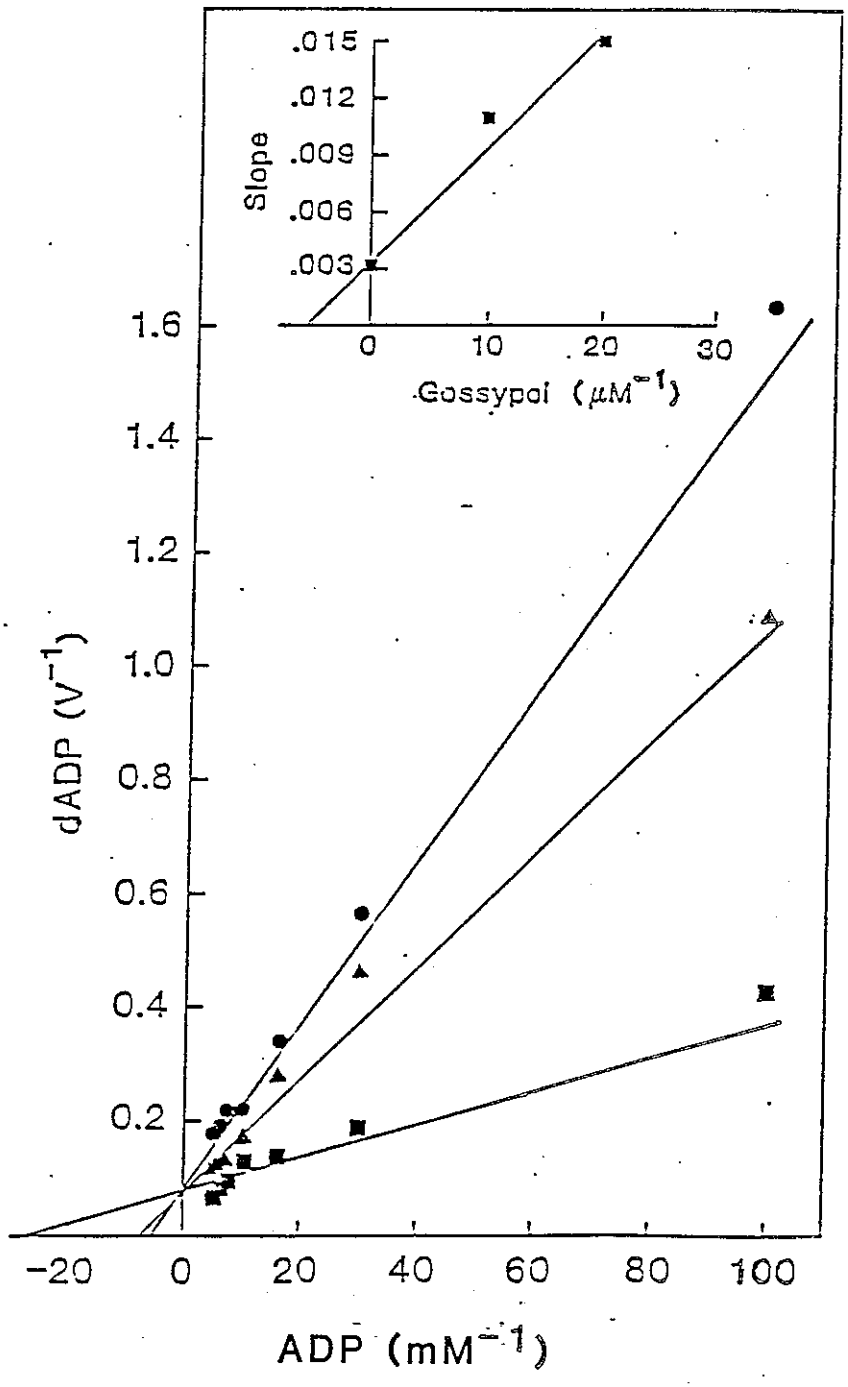

.012
.009
.006
.003
Slope
0
10
20
Gossypol (μM)
0.95
0.85
0.75
0.65
0.55
0.45
0.35
0.25
0.15
0.05
dCDP (V⁻¹)
-20
0
20
40
60
80
100
CDP (mM⁻¹)

Figure 20. Double reciprocal plots of velocity against ADP concentrations in mouse L cell extracts at several fixed concentrations of gossypol.

Activity in the absence (■) or presence of 10 μM (▲) and 20 μM (●) gossypol.

Inset: replot of slopes against drug concentrations.

Velocity = nmole dADP/mg/hr.


.015
.012
.009
.006
.003
Slope
0
10
20
30
Gossypol (μM^{-1})
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
dADP (V^{-1})
-20
0
20
40
60
80
100
ADP (mM^{-1})

DISCUSSION

Ribonucleotide reductase activity is cell cycle regulated and closely coupled to DNA synthesis. As cells enter S-phase of the cell cycle, ribonucleotide reductase activity is at its maximum and then decreases rapidly when DNA replication is completed. The structure of the enzyme is complex and the activity is allosterically regulated by nucleotide effectors (Thelander and Reichard, 1979; Wright, 1983, 1987). Inhibitors of ribonucleotide reductase can effectively affect DNA synthesis by reducing the supply of deoxyribonucleotides. One of the frequently studied inhibitors of the reductase is hydroxyurea which is a chemotherapeutic agent in the treatment of neoplastic diseases (Krakoff et al 1964; Bolin et al 1982). Hydroxyurea inhibits reductase activity by reducing the tyrosyl free radical of the M2 subunit (Atkin et al 1973; Kjoller Larsen et al 1982). Another inhibitor of ribonucleotide reductase activity is dATP, an allosteric effector which binds to the M1 subunit (Thelander et al 1980).

There have been many reports describing the isolation of variant cell lines that are resistant to inhibitors of ribonucleotide reductase (Wright, 1983; 1987; Wright et al, 1987b), and one of the frequently observed mechanisms is the overproduction of drug-sensitive activity (Lewis et al, 1978; Lewis and Wright, 1979; Akerblom et al, 1981; Lewis et al, 1983; McClarty et al, 1986a, 1987a; Choy et al, 1988). An additional increase in ribonucleotide reductase can occur when hydroxyurea resistant cells are cultured in the presence of hydroxyurea (Lewis and Wright 1979; Wright et al, 1980; McClarty et al, 1986a; 1987a). This characteristic was particularily pronounced in the highly drug resistant mouse cell line described in this thesis.

The LHF cell line exhibited only a modest elevation in enzyme activity (about 5 fold) when cultured in the absence of a selective agent, which was thought to be insufficient to generate a 30-fold greater than WT resistance to the drug in colony forming experiments. Furthermore, CDP and ADP reductase activities of WT and LHF cells demonstrated similar sensitivity to inhibition by hydroxyurea. Therefore, the high resistance of LHF cells could not be explained by the existence of a drug resistant form of the enzyme. In addition, unlike most other ribonucleotide reductase overproducing cell lines, M1 immunoprecipitation and M2 titration experiments showed that there was an increase in both M1 protein and M2 activity. The M1 subunit is not known to be a target of hydroxyurea. However, an overproduction of M1 subunit accompanying an increase in M2 activity was observed in the drug resistant cells. This suggested that the activity of the two subunits may be coordinately regulated. More recent studies showed that the level of M1 mRNA was elevated in the drug-resistant cells without detectable increase in gene copies (McClarty et al 1987a). This suggested that the regulation may occur at the level of transcription.

The question of why LHF^- cells were highly resistant to hydroxyurea but only contained about a 5-fold elevation in drug-sensitive ribonucleotide reductase activity was addressed by studying the characteristics of these cells during growth in the presence of the drug. It was found that LHF^+ cells contained at least 100-fold higher levels of M2 subunit activity. Also, the rate of ribonucleotide reduction was dependent upon the concentration of drug

in the culture medium. When grown in the presence of 5 mM hydroxyurea, the reductase activity increased by about 2.5 fold within 18 hours. The hydroxyurea induced elevation of ribonucleotide reductase activity was observed to require de novo synthesis of RNA and protein. When hydroxyurea was removed from the medium, the reductase activity in LHF cells decreased to only 33% of its original activity within 54 hours. Results from titration experiments showed that the level of M2 subunit activity declined by about 90% within 48 hours after hydroxyurea was removed from the medium. This behaviour of enzyme activity in LHF cells is clearly different from that of other ribonucleotide reductase overproducing cell lines (Wright, 1983, 1987; McClarty et al 1986a, 1987a). It appears that the LHF^- cells are able to modulate and drastically increase the level of reductase activity in the presence of hydroxyurea; and therefore they quickly become LHF^+ cells. This explains the observation that LHF^- and LHF^+ cells exhibited similar colony-forming abilities in the presence of increasing hydroxyurea concentration despite their apparent differences in enzyme activity.

After the completion of the study presented in this thesis, I have continued to participate in a more detailed investigation on a subclone of the LHF cell line (LHF-SC2) and more information on the molecular mechanism of drug resistance has been obtained (McClarty et al 1987a; Choy et al 1988). The LHF-SC2 cell line exhibits the same drug resistant characteristics as the parental LHF cell line (McClarty et al 1986b, 1987a). Monoclonal antibodies specific to the M1 and M2 subunits (Engstrom et al, 1984) have been used for immunoblot analysis

of cell-free preparations separated on a SDS-polyacrylamide gel. The results from these experiments showed that the quantity of both the M1 and M2 subunits were increased in the drug resistant cells. The immunoblot analysis also showed that there was a dramatic increase in the level of M1 and M2 subunits when the cells were grown in the presence of hydroxyurea and that this increase was dependent upon time of exposure and concentration of hydroxyurea. The evidence obtained by immunoblot analysis supported the results obtained by measuring the activity of ribonucleotide reductase. Furthermore, this study indicated that there was a quantitative increase in the cellular content of M1 and M2 subunits as suggested in this thesis.

Recently, Thelander and Berg (1986) have cloned the genes for both M1 and M2 subunits of murine ribonucleotide reductase. These cDNAs were used to elucidate the molecular changes that have occurred in the drug resistant cells (McClarty et al 1987a). The level of M1 and M2 messenger RNA (mRNA) in WT and LHF-SC2 cells was analyzed with the Northern blot technique. The results indicated a 2-3 fold increase in M1 message level and a 35-40 fold elevation in M2 message level in the LHF-SC2 cell line as compared to the WT cells. The levels of M1 and M2 messages did not change in response to the absence or presence of hydroxyurea in the growth medium.

The relative rate of transcription of the M1 and M2 genes was examined by using the nuclear run-off transcription assay in isolated nuclei of WT and drug resistant cells (McClarty et al, 1987a). The transcriptional activity of the M2 gene was about 2-3 fold higher in the LHF-SC2 cells than in the WT cells, and there was only a slight

increase in the transcriptional activity of the M1 gene in the LHF-SC2 cells.

Southern blot analyses were carried out on the genomic DNA of WT and LHF-SC2 cells (McClarty et al 1987a; Choy et al, 1988). The number of copies of the M1 gene was the same for WT and LHF-SC2 cells. However, the LHF-SC2 cells contained a 6 fold amplification in the number of copies of the gene for protein M2.

It is now established that the underlying molecular mechanism for drug resistance in the LHF cell line is through amplification of the M2 gene (McClarty et al, 1987a, Wright et al, 1987b). The increase in the number of copies of the M2 gene is probably the primary event that leads to the overproduction of the M2 subunit. However, the results obtained so far do not eliminate the possibilities that other secondary mechanisms such as increased translational efficiency and reduced turnover rate of the M2 protein may also have contributed to the drug resistant phenotype. These possible secondary events are presently being investigated.

The phenomenon of gene amplification in cultured mammalian cells has been widely reported to occur in many different drug resistant systems and it is estimated to occur at a frequency of approximately 10^{-4} to 10^{-7} per cell/generation (Schimke 1986; Stark 1986). Gene amplification has also been reported to occur in vivo, in human patients treated with methotrexate against neoplastic diseases. (Carman et al 1984; Horne et al 1984; Trent et al 1984). These observations have made the study of gene amplification significant in clinical applications. Recent studies with a series of clonally

related hydroxyurea resistant mouse cell lines, which were the progenitors of the LHF line, have suggested that gene amplification occurred very early in the selective process (Choy et al 1988). It appears that a combination of high frequency of occurrence and selective advantage have made gene amplification a predominant event in the selection of drug resistant variants. Another recent report from our laboratory has shown that the loss of M2 gene amplification in two cloned reverants of a hydroxyurea resistance Chinese hamster ovary cell line (CHO) was correlated with the decline of ribonucleotide reductase activity and reduced resistance to hydroxyurea (McClarty et al 1987b). This finding supports the view that M2 gene amplification played an important role in hydroxyurea resistance.

Among many drug resistant systems, methotrexate (MTX) resistance is probably the most intensively studied. Methotrexate resistant variant cell lines from a variety of cell types have been isolated and studied in detail. Four different mechanisms through which cultured cells acquired resistance to methotrexate have been described: (1) production of dihydrofolate reductase (DHFR) with less affinity for MTX (Flintoff et al 1976; Haber et al 1981); (2) an alteration in MTX transport (Sirotnak et al 1981); (3) overproduction of DHFR protein as a result of a proportional amplification of the DHFR gene (Alt et al 1978); and (4) production of a more active DHFR protein (Dedhar et al 1985). It appears that the LHF cell line and some MTX resistant cell lines share at least one common mechanism of drug resistance; that is the overproduction of drug sensitive enzyme through gene

amplification. Experimental evidence presented in this thesis suggested that the other three mechanisms of drug resistance observed in some MTX resistant cell lines were not involved in the development of hydroxyurea resistance of the LHF cell line.

Domin et al (1982, 1983) have described a MTX resistant human KB cell line with 40-fold overproduction of DHFR protein which would increase enzyme level by a further 5-fold when cultured in MTX supplemented medium. This particular response to MTX, the selective agent, is similar to the response of LHF cells to the presence of hydroxyurea. In the KB cell line, drug dependent elevation was blocked by cycloheximide but was not affected by actinomycin D. It was suggested that modulation of DHFR level by drug in the KB cells occurred at the level of translation. However, hydroxyurea induced elevation of ribonucleotide reductase activity in the LHF cells was found to be inhibited by both cycloheximide and actinomycin D. This suggested that continuous transcriptional and translational activities were necessary for the elevation of enzyme activity in response to hydroxyurea; therefore the LHF cell line was clearly different from the MTX resistant KB cell line.

It is known that hydroxyurea arrests cells at early S phase of the cell cycle (Ashihara and Baserga 1979). Therefore, it is possible that when LHF cells are cultured in the presence of hydroxyurea, a larger proportion of the cell population would be blocked in early S phase leading to the detection of higher ribonucleotide reductase activity. In order to examine this possibility, the cell cycle distribution of LHF cells grown in the

absence and presence of hydroxyurea were analyzed by the flow cytometric method. The results revealed that the proportion of cells in S phase in the two cell populations were essentially the same (McClarty et al 1987a). Perhaps this is not too surprising since LHF cells can grow normally in hydroxyurea supplemented medium.

The mechanism by which MTX and hydroxyurea trigger the further 5-fold increase in enzyme activities in their respective cell lines grown in the presence of drug is unknown (Domin et al 1982, 1983; McClarty et al, 1986a, 1987a). One possible mechanism for the induction of ribonucleotide reductase activity is through the intracellular nucleotide pools, since this enzyme plays a role in nucleotide metabolism, and the enzyme is known to be regulated by ribonucleotides and deoxyribonucleotides (Thelander and Reichard 1979). a Such regulatory role has been postulated for polyamine pools in the control of ornithine decarboxylase expression (Kahana and Nathans 1985; McConglogue et al 1986).

The hydroxyurea induced elevation of enzyme activity has been shown by titration experiments (Figure 16) to involve a dramatic increase (at least 10 fold) in M2 subunit activity. Immunoblot analysis indicated that the level of both M1 and M2 subunits were increased about 10-fold and 6-fold respectively when cultured in the presence of hydroxyurea (McClarty et al 1986a). It is conceivable that more M2 subunits were produced by the cells to overcome the inhibitory effect of hydroxyurea. However, the increase in the level of the M1 subunit was unexpected because it is not the site of action of hydroxyurea. Moreover, in wild type cells, the M2 protein is

normally the limiting subunit and the cell-cycle dependent expression of the M2 subunit regulates ribonucleotide reductase activity during the cell-cycle (Eriksson et al 1984; Engstrom et al 1985). We postulated that when the LHF cell line was grown in the presence of hydroxyurea there was a further overproduction of the M2 subunit, but a large proportion of these M2 proteins were inactivated by hydroxyurea and did not contain a tyrosyl free radical (McClarty et al 1987a). In E. coli, it has been demonstrated that an inactive B2 subunit, without a tyrosyl free radical, could still combine with the B1 subunit to form an inactive holoenzyme (Brown et al 1969; Larsson and Sjoberg 1986). So, in the presence of hydroxyurea, the large amount of inactive M2 protein in the LHF cells were actually competing against the active M2 proteins for the available M1 subunits. This created a situation where there was a shortage of active holoenzyme and the limiting subunit was the M1 protein. In order to attain an adequate level of ribonucleotide reductase activity it became necessary for the cell to also increase the level of the M1 subunit.

In brief, the drug resistance study presented in this thesis can be summarized as follows. The selection process of increasing the concentration of hydroxyurea in a stepwise manner has resulted in a cell population that is 30-fold more resistant to hydroxyurea (LHF) than WT cells. The drug resistant phenotype can be attributed to an overproduction of drug sensitive ribonucleotide reductase activity. The levels of both M1 and M2 subunits were found to be elevated. The LHF cell line showed a basal 5-fold increase in ribonucleotide

reductase activity. An additional 5-fold increase in enzyme activity was observed when the LHF cells were cultured in hydroxyurea supplemented medium. The hydroxyurea induced increase in enzyme activity was inhibited by actinomycin D and cycloheximide, suggesting that continuous de novo synthesis of M1 and M2 proteins and their corresponding mRNAs were necessary. This preliminary characterization of the LHF cell line has provided the foundation for a detailed analysis of the molecular events involved in the development of drug resistance (McClarty et al, 1987a; Choy et al, 1988; Wright et al, 1987b).

The level of ribonucleotide reductase activity in WT cells is low and difficult to detect. This low level of activity often hampers the study of the biochemical properties of the enzyme. The LHF cell line has been shown to overproduce ribonucleotide reductase activity that is similar to the WT enzyme with respect to enzyme kinetics and sensitivities to inhibitors such as dATP and hydroxyurea. These properties make the LHF cell line an invaluable source of ribonucleotide reductase enzyme for the study of the inhibitory effect of gossypol and other compounds (McClarty et al 1985; 1986b). The results showed that gossypol was a potent inhibitor of ribonucleotide reductase. The K_i values were determined to be about 1.72 M and 5.5 M gossypol for CDP and ADP reduction respectively.

A survey of the ID_{50} values (concentration of drugs required to reduce the enzyme activity by 50%) of some commonly studied inhibitors revealed that gossypol is about 60-fold more effective than hydroxyurea as an inhibitor of ribonucleotide reductase activity. The

Table 4. A comparison of ID_{50}[a] values of some commonly studied inhibitors of ribonucleotide reductase.

Inhibitors	ID_{50} (μM)	Subunit Inhibited	References
Gossypol	8	Not known	McClarty et al (1985)
Hydroxyurea	500	M2	McClarty et al (1986a)
Guanazole	1200	M2	Lewis and Wright (1974)
IMPY[b]	800	M2	Cory and Fleischer (1980)
MAIQ[c]	1.5	M2	Sato et al (1984)

[a] ID_{50} is the concentration of inhibitor required to reduce the enzyme activity by 50%. CDP was used as the substrate in these experiments.

[b] pyrazolo-imidazole (IMPY, NSC 51143).

[c] 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ, NSC 92188).

potency of gossypol is comparable to 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ, NSC 246112) (Table 4). MAIQ is a M2 subunit specific inhibitor (Cory and Fleischer, 1979) and it is the most potent ribonucleotide reductase inhibitor described to date. In the light of these results the inhibition of DNA synthesis by gossypol previously reported by Wang and Rao (1984) was, at least in part, due to the inhibition of ribonucleotide reductase.

Recently, Rosenberg et al (1986) demonstrated that gossypol also inhibited the function of DNA polymerase α and β from mammalian cells. The sensitivity of DNA polymerase α to gossypol inhibition was similar to that observed for ribonucleotide reductase in this study. However, the mechanism by which gossypol inhibited ribonucleotide reductase and DNA polymerase α appeared to be distinctively different. Inhibition studies (Figures 19 and 20) suggested that gossypol inhibited ribonucleotide reductase activity in a competitive manner whereas DNA polymerase α was reported to be inhibited non-competitively by gossypol (Rosenberg et al 1986). The exact mechanism by which gossypol inhibits these enzymes is unknown. Since gossypol inhibits ribonucleotide reductase competitively and its chemical structure is similar to those of the purine and pyrimidine moieties of nucleotides; it can be speculated that gossypol acts as a nucleotide analog.

The anti-proliferative effect of gossypol on cultured cells in vitro is likely to be through the inhibition of at least two important DNA replicative enzymes. The antifertility effect of gossypol in vivo

may occur through another mode of action. It has also been recently found that gossypol specifically inhibited a lactate dehydrogenase isoenzyme (LDH-X) found only in germ cells (Morris et al 1986). This interesting observation suggested that gossypol affected spermatogenesis through the inhibition of testicular LDH-X. Clearly, a thorough understanding of the action of gossypol is important because of its potential to be used as a male antifertility drug (Segal 1985) as well as an antitumor agent (Tso 1984; Rao et al 1985). The work in this thesis documents at least one important site of action for this compound.

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